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THE COMPARATIVE ANATOMY AND MORPHOLOGY OF THE FLOWERS AND INFLORESCENCES OF THE PROTEACEAE —II. SOME AMERICAN TAXA*

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Introduction

In a previous paper the author described and discussed the comparative morphology and anatomy of thirty-one species from eighteen genera of the family Proteaceae found in Australia (Haber, 1959). The floral anatomy of the Australian species studied indicates that the proteaceous flower is highly specialized in an advanced stage of reduction. The ancestral inflorescence was probably a panicle, but phylogenetically reduced to the extent that only twin or single flowers remain on the tertiary branch systems of the inflorescence axis. Vascular investigations give evidence that the ancestral flower is dichlamydeous and that reduction was the factor for the present apetaly. Among the Australian genera some of the flowers still retain their primitive characteristics, while others exhibit more advanced ones. The general evolutionary trend seems to have led to apparent simplification through reduction, resulting in more complexity and compression of the flowers with concomitant cohesion and adnation of floral organs and their vascular tissues.

The aim of this study was to determine whether a detailed anatomical study of American[1] species might reveal additional evidence for the above conclusions; also clarify the nature of the glands, discs and scales; interpret zygomorphy and finally strengthen the conclusion that the Proteaceae may not be allied with the Apetalae.

The Proteaceae are divided into two sub-families: the Persoonioideae, which are not represented in America; and the Grevilloideae, abundantly found in Australia with several species in Central and South America (Engler & Prantl, 1894). The Grevilloideae are subdivided into three tribes, only two of which, the Grevilleae and Embothrieae are represented in Central and South America. The genera *Euplassa, Roupala, Panopsis, Guevina* and *Embothrium* are purely American genera, while *Grevillea, Lomatia, Euplassa,* and *Embothrium* are found in Australia (Sleumer, 1954). Africa does not possess a single genus endemic to Central and South America and Australia.

Materials and Methods

Inflorescences as well as flowers in bud and in anthesis of twenty-three species comprising seven of the nine genera found in America were studied. Herbarium specimens were loaned by the Chicago National Museum through the courtesy of Dr J. A. Steyermark[2]. *Roupala pseudocordata* flowers were obtained from Bailey Hortorium, Cornell University. Inflorescences of *Grevillea robusta* and *Roupala complicata*, preserved in 70 per cent alcohol, were collected by Señor San Ramon of the Ministry of Forestry, Costa Rica, and sent by Professor Daniel E. Alleger of the University of Florida, Agricultural Mission to Costa Rica.

1. The word American here refers only to the species of Central and South America. No indigenous species are found in North America.

2. Specimens numbered indicate the loaned Chicago Museum species.

Herbarium specimens were soaked in distilled water for three days, boiled from three to five minutes in a 2 per cent solution of potassium hydroxide, washed overnight in running water, and then treated as preserved material. All specimens were dehydrated, imbedded in tissuemat, cut serially at 10 microns and stained with crystal violet and erythrosin. Both longitudinal and cross sections were cut, but the former served only as checks. At least three, sometimes as many as six or eight flowers of a species were sectioned and studied.

The list of specimens examined follows: *Grevillea banksii* R. Br. (Guatemala, No. 1,095,649); *G. robusta* A. Cunn. (Mexico, No. 464,956, also from Costa Rica); *Roupala pseudocordata* Pitt. n. sp. (Caracas) Cornell University; *R. montana* Aubl. (Costa Rica No. 855,400), *R. complicata* H. K. B. (Costa Rica); *R. glaberrima* Pittier. (Costa Rica, No. 1,350,740); *Panopsis suaveolus* (Kl. and Karsten) Pittier (Panama, No. 1,004,850); *Guevina avellana* Mol. (Chile, No. 649,469); *Euplassa nebularis* Rambo et Sleumer (Brazil, No. 1,403,191); *E. pinnata* (Lam) Sleumer (French Guiana, No. 639,243); *E. rufa* Sleumer (Rio Janeiro, No. 668,118); *E. goyazensis* K. Schum. (Brazil, No. 894,262); *E. isernii* Cuatri (Peru, No. 876,380); *E. obversiflora* (Mez) Steyermark. (Brazil, No. 538,753); *Embotyrium weberbaueri* Perk (Peru, No. 1,316,543; No. 736,771); *Embothrium mucronatum* Willd. ex Roem & Schult. (Ecuador, No. 704,875); *E. grandiflorum* Lam (Ecuador, No. 1,237,680); *E. coccineum* Forst. (Argentina, No. 1,294,180); *Lomatia hirsuta* (Lam) Diels (Ecuador, No. 1,280,287); *L. ferruginea* R. Br. (N. Valdivia No. 633,773); *L. dentata* R. Br. (Chile, No. 663,973); *L. obliqua* R. and Par. (Chile, No. 639,431).

Abbreviations

AN, anthers; *BR*, bract; *C*, carpellary traces; *CX*, calyx; *DC*, dorsal carpellary; *E*, style; *F*, filaments; *G*, gland; *GB*, gland bundle; *GT*, gland traces; *GY*, stipe or gynophore; *I*, inflorescence axis; *LS*, lateral sepal; *MS*, median sepal trace; *N*, connective; *O*, ovule; *OV*, ovary; *P*, pedicel;

PC, pollen-collecting apparatus, *PD*, peduncle; *R*, rachis; *SA*, scale; *SCL*, sclerenchyma fibers; *SL*, slit for style; *SM*, stigma; *TR*, transmitting tissue; *VC*, ventral carpellary; *X*, secondary axis.

Floral Anatomy

I. GREVILLOIDEAE — Flowers in pairs with single deciduous or abortive bract or with an involucre of imbricated bracts; inflorescence anomalous; ovules 2, 4, or many.

GREVILLEAE — Inflorescences generally axillary or terminal, in dense or lax racemes, with deciduous or abortive bracts, rarely with involucre of bracts. Flowers actinomorphic or zygomorphic. Receptacles often oblique, hence the organs of a single whorl do not arise at the same level. The hypogynous glands: two, three, or four distinct fleshy lobes, a cup or semiannular ring in which one or two anterior lobes situated on the placentary side of the gynoecium, become suppressed. Ovary, sessile or stipitate, unilocular; ovules, two or four collateral, amphitropous, anatropous or orthotropous.

Since most of the species of this tribe have the same fundamental pattern of vascularization, a detailed description of a single species of each genus will suffice. Deviations from basic plan will be noted.

Grevillea, the largest genus in the family, is decidedly endemic in Australia. *Grevillea robusta* and *G. banksii*, introduced in both North and Central America, are now commonly found as ornamental trees in parks and lawns in California, Florida and Central America, and as shade trees among coffee plantations in the latter area (Standley, 1931, 1937; Standley & Steyermark, 1946).

Grevillea robusta A. Cunn.— This species has been investigated anatomically by Brough (1933) and Kausik (1941). Since the author received specimens from Mexico and Costa Rica, it was decided to examine transverse sections of the flower for possible morphological and anatomical variations.

In most respects the type of inflorescence and floral anatomy of the writer's specimens of *G. robusta* coincided with the vascular behavior recorded by Brough (1933), Kausik (1938, 1941), and Rao

(1957). However, the author finds in both Mexican and Costa Rican specimens that double stamen traces are present. These paired traces continue slightly above the ovary level, then fuse within the filament, and continue into the connective. Thus the single stamen trace represents the union of the double stamen strands. The horse-shoe shaped gland (Fig. 6, *G*) receives at its base some vascular elements derived from traces of the lateral sepal bundles (*LS*) in its vicinity. These basal bundles send branches into the lower half of the gland (Figs. 6, 7, *G*). Branches from these traces (Fig. 5, *C*) augment also the vascular ring in the monocarpel. Both Kausik (1941) and Brough (1933) stated the gland to be non-vascular in *Grevillea robusta.* Rao (1957) reports that " in the same species of *Grevillea* plus fifteen other species of *Grevillea,* the nectary was profusely vascularized ". However, Kausik (1941) concluded " the nectary is equivalent to a reduced corolla on the verge of extinction ", but Rao believed this interpretation as " untenable ". Brough stated that since no vascular tissue was evident there was " no ground for interpreting the scales (glands) as the morphological equivalent of a reduced perianth. It would seem then to be merely an adventitious accessory development of the torus ".

This author believes that the alterni-sepalous position of the glands (Fig. 8, *G*) and the origin of their vascular bundles from the lateral sepal bundles warrant the conclusion that the three-lobed gland is a vestigial corolla, the anterior lobe of which has become suppressed through reduction.

Grevillea banksii features simple racemes, septate hairs on the flowers, weaker strands penetrating the base of the glands, the absence of double stamen traces, amphitropous ovules laterally attached about the middle. Otherwise its vascular supply corroborates that of *G. robusta.*

The special features of this genus are: the slightly zygomorphic flowers; the alterni-sepalous position of the glands; the
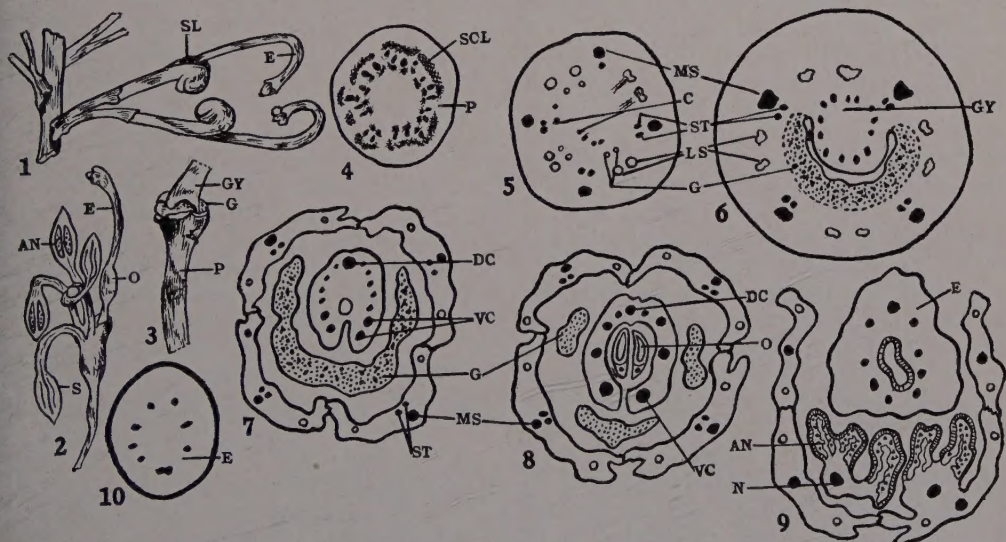
Figs. 1-10 — *Grevillea robusta* A. Cunn. Fig. **1.** Secondary axis of infloreseence showing a pair of flowers. × 1. Fig. **2.** A single twin flower at anthesis. × 1. Fig. **3.** Pedicel showing semicircular gland and origin of gynophore. × 2. Figs. **4-10.** C.s. × 15. Fig. **4.** Eustele in pedicel. (Bundles capped by sclerenchyma, not shown in following sections.) Fig. **5.** Base of oblique receptacle showing arrangement of various bundles. Fig. **6.** Oblique receptacle at base of gland (larger dots in gland represent vascular tissue). Fig. **7.** Free gynophore surrounded by semicircular gland. Fig. **8.** Ovular level showing two collateral ovules, and the three free lobes of gland. Fig. **9.** One pair of anthers and expanded stigma. Fig. **10.** Style.

origin of the gland vasculature from the lateral sepal bundles, the three-lobed semi-annular gland, the anterior lobe of which is suppressed; the presence in some species of double stamen traces (Figs. 5-8), originally conjoined to the median-sepal bundle; and a stipitate ovary.

Roupala complicata H. B. K.— *Roupala* is a purely American genus occurring in mountainous regions. The inflorescence is an axillary raceme. The small, geminate, actinomorphic flowers with four scales (glands) possess distinct pedicels. The latter arise from short peduncles imbedded wholly in the cortex of the rachis. The peduncles account for the ridged and furrowed outline of the rachis of the inflorescence (Figs. 11-19).

The base of the rachis is usually composed of a ring of many irregular-sized discrete bundles. These collateral bundles are almost encircled by heavy sclerenchyma fibers (Fig 13, *SCL*). At a higher level where flowers originate, a continuous ring with little parenchyma between the individual bundles is formed. At the nodes, in the ridges, branch traces inaugurate the peduncular steles (Figs. 15-18, PD_1, PD_2). These steles divide radially to form twin pedicellate steles (Fig. 18, PD_2, P). The latter steles are composed of eighteen or twenty bundles, which possess well developed xylem elements, supplying the various organs of the flower (Fig. 20).

The four median sepal traces diverge first from the receptacle. Eight lateral sepal bundles are arranged in pairs between the median sepal bundles (Fig. 21, *MS*, *LS*). Thus twelve bundles supply the calycine whorl. The calycine bundles divide tangentially as usual, resulting in four midrib bundles for the sepals and four stamen bundles. The outer half of each lateral sepal bundle supplies the margin of a sepal, the inner half, the scale (gland) (Fig. 21, *LS*, *SA*). These organs are hypogynous, free-lobed for greater depth and non-glandular. These vascular strands do not penetrate the depth of the scale, but terminate shortly above its base (Fig. 23, *SA*). The remainder of the bundles in the receptacle plus residual tissue from the lateral sepal-gland trace continues into the gynoecial area. Hence the lateral sepal bundle is a fused one: a lateral sepal-gland-carpellary bundle. The base of the ovary wall contains many bundles (Fig 24). A dorsal with two lateral branches and two ventral bundles persist and continue into the style (Figs. 27, 28, *E*). Two pendulous, orthotropous ovules, each arising from the tip of the loculus are vascularized by branches from the ventral bundle nearest it (Fig. 24). Above the level of the origin of the ovules, the loculus of the ovary is continuous with the exterior through an opening on the adaxial side of the carpellary wall between the two ventral bundles (Figs. 24, 26). This suggests a modified conduplicate carpel open at the base with sub-marginal ovules. The broad filaments, inserted above the middle of the sepals, are traversed by a single bundle, derived from the median-sepal bundle in the receptacle (Figs. 21, 22, 25, *ST*). Anthers are free at a higher level (Fig. 28, *AN*). Pollen is triporate. The style is straight and fusiform, but devoid of a pollen-collecting device. Only the dorsal bundle reaches the clavate stigma.

The vascularization of three other species studied conformed closely to that of *R. complicata*. *R. glaberrima* has shorter pedicels and its axillary and terminal racemes are glabrous. In *R. montana* the axillary inflorescences resemble spikes, the pedicels are longer, the flowers tomentose and the ovary with long hairs.

The most noteworthy features are: the actinomorphic flowers, four symmetrical scales supplied basally by vascular strands from the usual lateral sepal bundles, short apiculated connectives (Fig. 29, *N*); two orthotropous, pendulous ovules and an open carpel at its base. The peduncles of the twin flowers form part of the inflorescence axis (Figs. 11, 12, *PD*).

Panopsis suaveolus Kl. and Karsten — The genus *Panopsis* is endemic to Central America. The panicled inflorescence bears geminate flowers on both terminal and axillary racemes (Fig. 30). The large eustele of the rachis axis is composed of many discrete bundles protected externally and internally by the heavy-walled fibers (Fig. 32, 32_1, *SCL*). Branch traces produce the peduncular stele, which is imbedded in the cortex of the rachis. A bract subtends the peduncle (Fig. 32, *BR*).
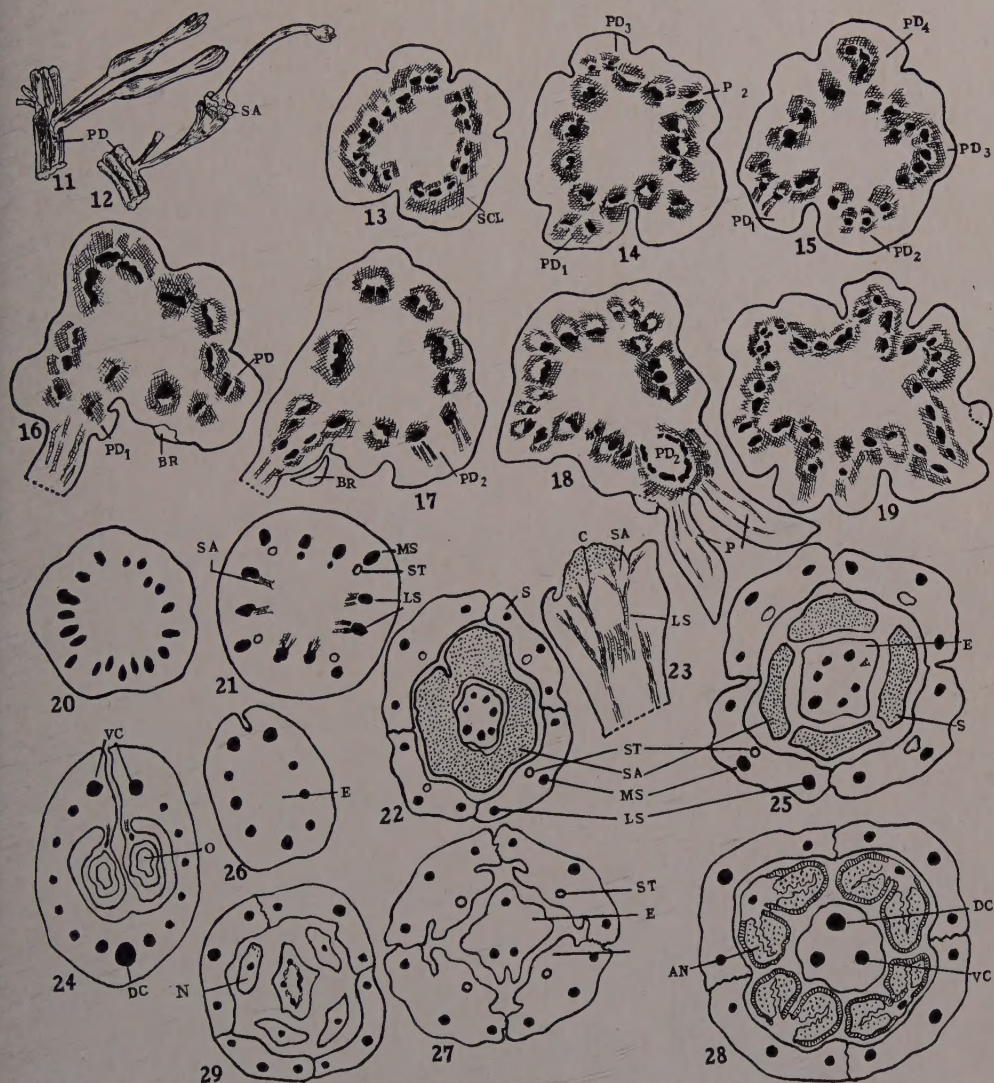
Figs. 11-29 — *Roupala complicata* H.B.K. Fig. 11. Buds arising from furrowed rachis. × 2. Fig. 12. Single twin flower with four scales and carpel (calyx removed). × 2. Figs. 13-29. C.s. showing the vascular behaviour of inflorescence axis and one flower (except Fig. 23). Fig. 13. The rachis eustele; bundles almost surrounded by sclerenchyma fibres. × 25. Fig. 14. Three pairs of bundles serving as branch traces. × 20. Fig. 15. Beginning of peduncular steles, *PD* 1-3. × 20.   Fig. 16. Formation of one of twin pedicels from PD_1. × 20. Fig. 17. Peduncle 2 originating from branch traces. × 20.   Fig. 18. Peduncular stele 2 almost complete, twin pedicels formed. × 15.   Fig. 19. A node of rachis subtended by bract; origin of several peduncles. × 15. Fig. 20. Pedicellate stele. × 20.   Fig. 21. Receptacle with bundles organized in position for the various organs of the flower. × 20. Fig. 22. Sepals separated from scales. × 33. Fig. 23. L.s. showing distribution of bundles at the tip of receptacle. Fig. 24. Ovary, two pendulous ovules in open carpel. × 70. Fig. 25. Lobes of scales alternating with sepals. × 38.   Fig. 26. Style slightly open. × 40. Fig. 27. Filaments adnate to sepals. × 35.   Fig. 28. Anthers with broad connectives. × 28. Fig. 29. Large apiculate connectives. × 23.

After a slight elongation of the peduncle, its vascular cylinder constricts and results in the two pedicellate eusteles (Fig. 33, PD_1, PD_2). In the receptacle the various bundles in each pedicel become arranged to conform with the position of the various organs (Figs. 34, 35). The outer whorl of bundles constitute the median-sepal bundles (Fig. 35, MS). Their tangential division forms the midrib sepal bundles and stamen bundles (Fig. 35, MS, ST). The next inner whorl constitutes the four lateral sepal bundles, which give rise to the marginal traces (Fig. 34, LS), of adjacent sepals. From these depart weak gland traces discernible only to the base of the four-lobed disc or cup (Figs. 35, 36, G).

Here also glandular traces depart to the pith to supply the gynoecial ring (Fig.35, C). Hence the triple-fused lateral sepal bundle supplies three organs of the flower: the sepal, gland and carpel. The carpellary wall is supported for its greater length by the dorsal and two ventral bundles (Figs. 37-44, VC, DC). At the tip of the loculus of the ovary, which is open basally, two collateral orthotropous, pendulous ovules receive the ovular traces from the nearest ventral carpellary bundle (Figs. 39-41, VC). The broad filament (Figs. 39, 42, F) affixed below the middle of the sepal receives a strong trace, which continues into the apiculate connective (Fig. 44, N). Pollen is triporate.
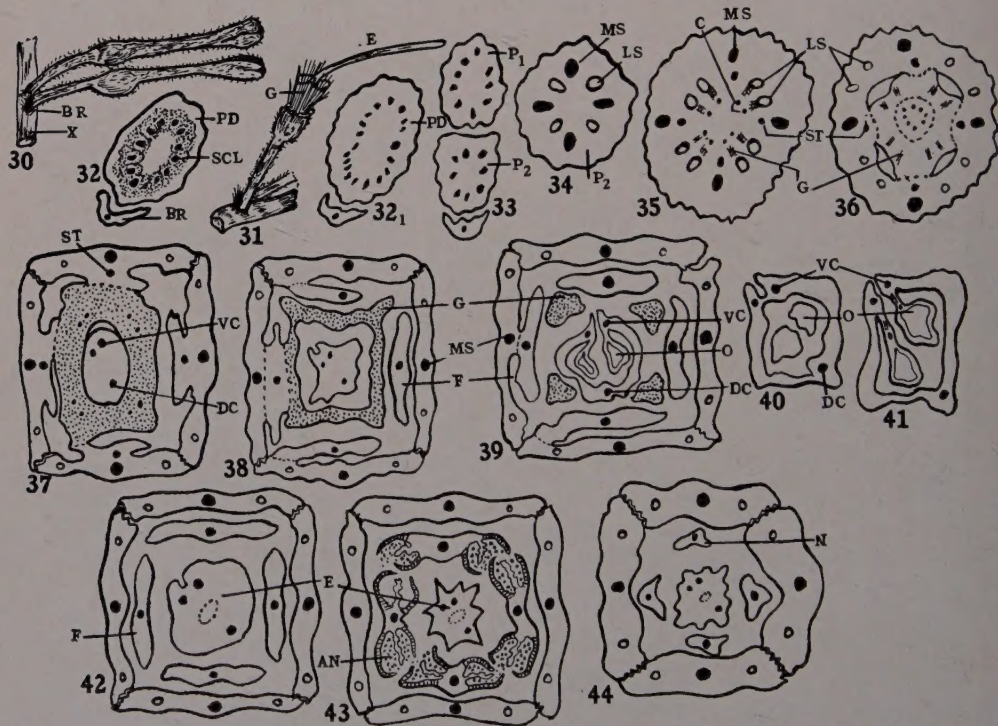
FIGS. 30-44 — *Panopsis suaveolus* Kl. & Karsten. Fig. 30. Geminate buds. × 3. Fig. 31. Single flower (calyx removed) showing cupulate gland and style. Fig. 32. Peduncle with bract; sclerenchyma surrounding bundles (not shown hereafter). × 10. Fig. 32_1. Elongation of peduncle. × 18. Fig. 33. Pedicels separated. × 20. Fig. 34. Single pedicel showing arrangement of sepal bundles. × 30. Fig. 35. Receptacle showing gland traces and carpellary traces. × 20. Fig. 36. Separation of calycine whorl from gland. × 20. Fig. 37. Gradual separation of gland and filament from calyx. × 24. Fig. 38. Gland almost free from calyx. × 24. Figs. 39-44. × 24. Fig. 39. Two collateral, pendulous ovules, tips of glandular lobes. Fig. 40. Ovary with two ovules. Fig. 41. Ovules attached to sub-margins of open ovary. Fig. 42. Free filaments, and style. Fig. 43. Anthers free. Fig. 44. Connective appendages.

Noteworthy features in this genus are: four-lobed cupulate disc supplied basally only with bundles from the lateral sepal bundles, the open suture of the carpel, the apiculate connectives, and two ovules.

Guevina avellana Mol.— The dissected siphonostele of the rachis is composed of groups of large vascular bundles with groups of smaller bundles interspersed (Figs. 47-48). The xylem is quite compact in radial rows with little parenchyma between the bundles. The entire eustele is capped heavily, both externally and inwardly, by heavily-walled sclerenchyma

fibers (Fig. 47, *SCL*). This sclerenchyma tissue is very common in varying degrees in most American species examined. The smaller bundles originate the branch traces (Fig. 48, *PD*) which multiply to form the usual secondary eustele for the peduncle (Fig. 49, *PD*). Tangential elongation, constriction and subsequent separation of the peduncular stele (Figs. 50-52, *PD*) result in the usual steles for the short pedicels of the geminate zygomorphic flowers (Fig. 53, P_1, P_2). Externally these pedicels apparently seem conjoined basally, but the line of demarcation
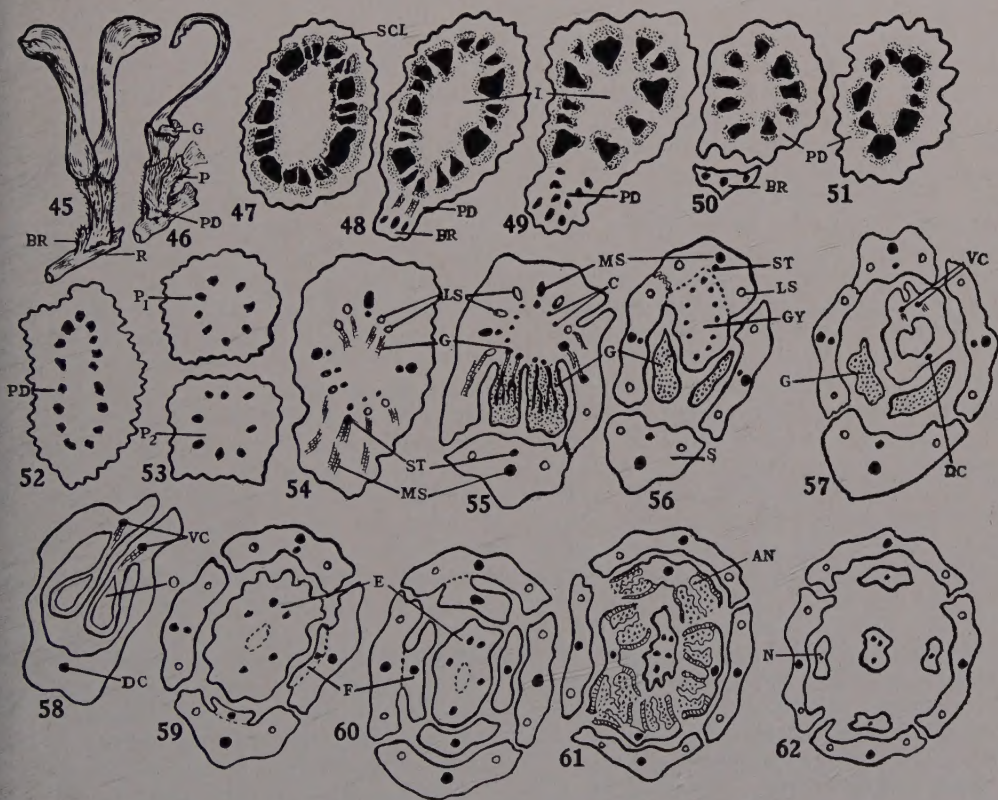
FIGS. 45-62 — *Guevina avellana* Mol. Fig. 45. Geminate flowers in bud. × 3. Fig. 46. Single flower showing gland and carpel. × 3. Fig. 47. C.s. rachis of an inflorescence. × 13. Fig. 48. Origin of bract and branch traces for peduncle. × 13. Fig. 49. Formation of peduncular stele. × 13. Fig. 50. Separation of bract and complete peduncle. × 20. Fig. 51. Elongation of peduncle. × 40. Fig. 52. Construction of peduncular eustele. × 20. Figs. 53-56. × 30. Fig. 53. Two pedicels separated. Fig. 54. Pedicel at base of oblique receptacle showing distribution of bundles. Fig. 55. Two glands and their vasculature. Fig. 56. Tip of glands and gynophore. Fig. 57. Tips of glands and slightly opened ovary. Fig. 58. Open ovary with two pendulous collateral ovules. × 60. Figs. 59-62. × 30. Fig. 59. Style and sessile filaments. Fig. 60. Some free filaments. Fig 61. Anthers free. Fig 62. Connectives and style within calyx.

is evident microscopically. The oblique receptacle reveals the organs of a single whorl. These organs originate at different levels (Fig. 54). The perianth parts on the adaxial side of the receptacle arise before those on the abaxial side at a node. At the base of the receptacle, the vascular ring is composed of eight bundles (Fig. 53, P_1, P_2). Four represent the usual median sepal bundles, a fusion of dorsal bundle and the adnate stamen bundle (Fig. 54, MS, ST). The remaining four represent fused lateral sepals or commissural bundles. Later, radial chorisis resolves them into the right and left marginals of adjacent sepals (Fig. 54, LS).

The most unique feature of this genus is the development of only two glands fused basally (Figs. 55, 56, G). Their vascular supply originates from the commissurally divided bundles nearest them, then enter the base of the gland, continuing as short ramifications within the gland (Fig. 55, G). Since two anterior lobes of the gland are absent, their traces form part of the supply for the open, modified conduplicate gynoecium with a stipitate, hirsute ovary (Fig. 55, C). Basally one dorsal, two ventrals and two branches of each penetrate the ovary wall. These strands increase in number above the base of the gland. The dorsal branches persist for a short distance, but fade out in the style, the main dorsal only remaining. The laterals discontinue (Fig. 57, VC, DC). Each ventral sends a branch to the nearest of the two collateral, pendulous, orthotropous ovules attached apically to the loculus (Fig. 58, O). The style is strongly curved. The stigma, bearing a pollen-collecting device below is oblique. The stamen bundles of the receptacle continue in the broad filaments (Figs. 59, 60, F) and the connective appendages (Fig. 62, N). The pollen is triporate.

The specializations observed in this genus are: the zygomorphic flowers, the two-lobed gland, the absence of the two anterior lobes of the gland, the stipitate ovary, the pollen-collecting device, connective appendages and triporate pollen.

Euplassa goyazensis K. Schum.— Six species of the purely American genus were studied. The origin of the pedicel and the subsequent behaviour of the vasculature

of the flower follows the general plan of the Grevilleae. A description of one species will present the salient conditions.

The inflorescences of *Euplassa* are largely narrow, raceme-like panicles. Flowers are small, slightly zygomorphic in some species, actinomorphic in the majority examined; floral tube is oblique, widened at the base, adnate to the ovary. Two collateral, orthotropous ovules develop at the apex of the stipitate ovary (Fig. 74).

The rachis of the inflorescence produces the peduncular steles (Figs. 65, 66). After elongation, and constriction, two eusteles result, each being the pedicellate stele of a geminate flower (Figs. 67, 68, P_1, P_2). In the base of the receptacle, two whorls of compound or fused bundles diverge toward the pith, each leaving behind a single stelar gap (Fig. 69, MS, LS). At a higher level the tangential separation of the bundles of the peripheral whorl results in the median or dorsal midrib of a sepal, and the stamen bundle (Fig. 71, MS, ST). On alternating radii, the four commissural bundles become disposed into the right and left marginals of adjacent sepals (Fig. 70, LS). From these, the four distinct glands (Figs. 71, 72, G) receive their traces which ascend distally for a short distance into those organs. Each alterni-sepalous gland (Figs. 73, 75) can be construed to be a petal, receiving its vascular tissue from the two lateral bundles of adjacent sepals. Glandular bundles are small with few xylem elements, but more phloem. The lateral sepal-gland traces send branches in the pith, and with isolated bundles in the receptacle ·continue into the stipitate ovary forming a gynoecial cylinder (Figs. 72, 73, C). At the ovular level, a single dorsal, two ventrals with their lateral branches are evident (Fig. 74, VC, DC). Each ventral serves as the origin of the ovular traces. The three main carpellary bundles continue into the simple style (Figs. 75-78) and into the club-shaped stigma devoid of a pollen-collecting apparatus. The stamens possess short filaments with the usual single trace. Anthers are sub-sessile with connective appendages (Fig. 76, AN).

Among the six species of *Euplassa* examined, the vascular pattern is generally
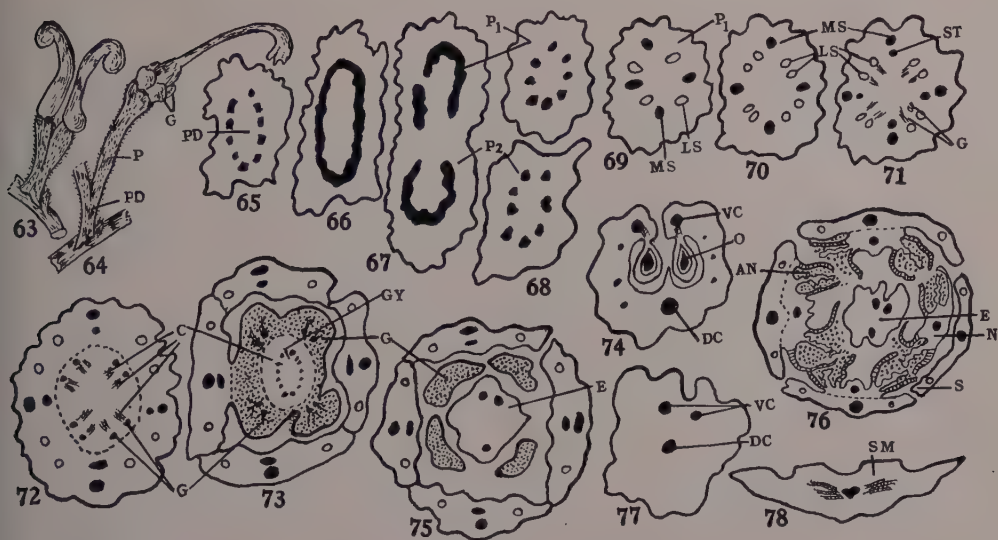
FIGS. 63-78 — *Euplassa goyazensis* K. Schum. Fig. 63. Geminate floral buds. × 2. Fig. 64. A single flower showing glands and carpel (calyx removed). × 2. Fig. 65. Peduncle. × 15. Figs. 66-74. × 18. Fig. 66. Peduncle elongated. Fig. 67. Eustele of peduncle constricted. Fig. 68. Geminate pedicels. Fig. 69. Pedicel with calycine whorl. Fig. 70. Arrangement of the calycine whorl in the receptacle. Fig. 71. Gland traces originating from lateral sepal bundles; stamen bundles from median sepal bundles. Fig. 72. Carpellary traces arising from gland bundles. Fig. 73. Separation of base of gland from calyx. Fig. 74. Ovary with two pendulous collateral ovules. Figs. 75-78. × 30. Fig. 75. Tips of glandular lobes and style. Fig. 76. Anthers with broad connectives. Fig. 77. Opened style. Fig. 78. Stigmatic area.

identical. In the inflorescences of *E. obversifolia* and *E. isernii*, the racemes of the panicle are quite long and compact; in *E. pinnata* and *E. nebularis*, the racemes are lax; the pedicels shorter with very small vascular bundles; *E. rufa* is few-flowered. *E. rufa* and *E. goyazensis* have ramifications of vascular tissue in the glands. *E. obversifolia* develops much sclerenchyma in its cortex.

Observations of note in this genus are: panicled inflorescences; both actinomorphic and slightly zygomorphic flowers, stipitate ovaries, two pendulous orthotropous ovules, four distinct glands with slight vascular ramifications, apiculate stamens, and triporate pollen.

II. EMBOTHRIEAE — This second tribe of the Grevilloideae is represented in America. The species studied belong to the genera *Embothrium* and *Lomatia*. Taxonomically they are separated from the Grevilleae by the presence of an involucre below the inflorescence, the arrangement of numerous ovules in two

or four imbricated series, winged seeds separated by a thin lamina, and triporate pollen.

Embothrium — It is largely a South American genus (Sleumer, 1954). The inflorescences are terminal racemes or thyrses, compact or lax; geminate zygomorphic flowers on strikingly furrowed axes, which are conjoined for some length one to another and to the rachis (Fig. 79, *PD*). This arrangement constitutes the axis of each terminal raceme and produces a much furrowed outline to the periphery.

Four species studied, show a general uniform pattern in their vascular behaviour. The base of the rachis of *E. weberbaueri* forms a compact eustele of a varying number of collateral bundles with the leaf gaps and interfascicular areas fairly clearly delimited one from the other (Fig. 91). Each of the more or less forty bundles at a higher level is capped internally and externally by a shallow crescent of heavy-walled fibers (Fig. 92). The same pattern occurs in *E. mucronatum*. However, the
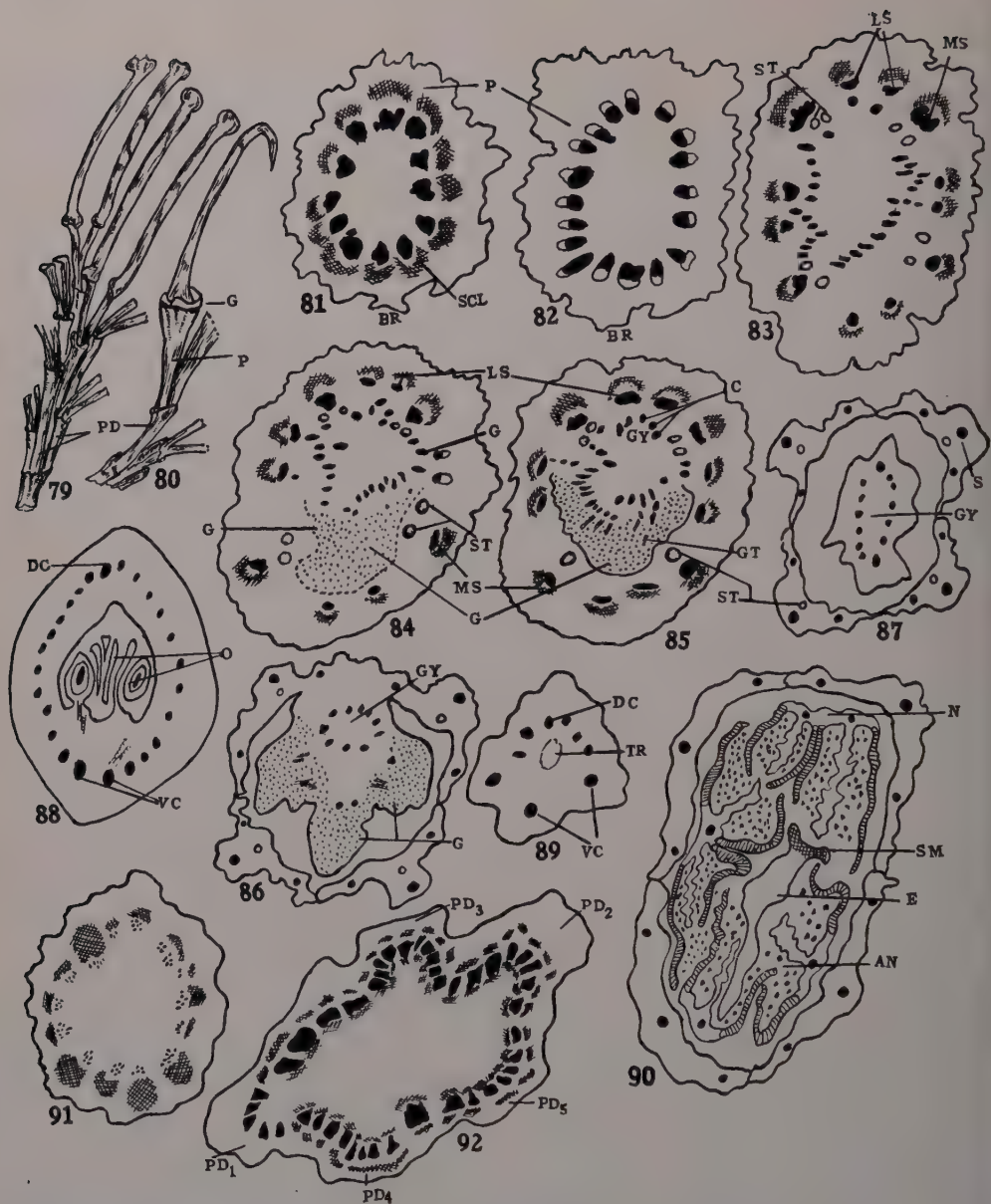
FIGS. 79-92 — *Embothrium* species. Fig. 79. *Embothrium grandiflorum* Lam., portion of inflorescence depicting conjoined peduncles with twin flowers. × 1. Fig. 80. *E. mucronatum* Willd ex. Roem & Schult., conjoined peduncles, single carpel surrounded by semi-circular gland. × 1. Figs. 81-90. *E. mucronatum*, c.s. at various levels to show behavior of vascular skeleton of pedicel and flower. Fig. 81. Base of single pedicellate eustele, bundles heavily capped with sclerenchyma fibers. × 40. Fig. 82. Pedicel with increased bundles, near receptacle. × 30. (No fibers evident). Fig. 83. Oblique receptacle with organized bundles to sepals, glands, paired stamens and carpel. × 30. Figs. 84-85. Tip of receptacle, appearance of gland, its traces, and the gynoecial vascular ring. × 25. Fig. 86. The three-lobed semi-circular gland, sepal free. × 15. Fig. 87. Calyx enclosing gynophore. × 15. Fig. 88. Ovary with several ascending ovules. × 25. Fig. 89. The style with reduced vascular supply. × 20. Fig. 90. Anthers free from calyx, curvature of style with stigmatic surface. × 13. Figs. 91, 92. *E. weberbaueri*. × 20. Fig. 91. C.s. rachis, below origin of flowers. Fig. 92. C.s. rachis at nodes for flowers. This nodal level portrays the vascular behavior of five peduncles, which will form complete peduncular steles at various levels, each giving rise to twin pedicels.

vascular ring is composed of a fewer number of bundles. As in the genus *Roupala*, at various nodal levels, branch traces depart from the rachis. These traces form the peduncular eusteles, which are imbedded or adhere vertically to the cortex of the rachis for some length (Fig. 92, PD_{1-5}). At the free terminus of each peduncle, the secondary axes form the free pedicels bearing the geminate zygomorphic flowers. These are subtended by a small bract (Fig. 81). The pedicellate eustele is composed of a varying number of vascular bundles, though eight is a more common number. These increase in extent (Figs. 81, 82). At the tip of the oblique receptacle, the number increases to supply the various organs, however, in very close whorls. These receptacle bundles become almost abruptly and simultaneously dissociated into traces (Fig. 83). Internodes are almost obliterated. Four outer bundles send traces to the calycine whorl. The next inner whorl of eight bundles is disposed in alternating pairs between the calycine bundles. All twelve calycine bundles are fused bundles. Each calycine bundle dissociates immediately into the usual sepal midrib bundle and two, instead of one, stamen bundles (Fig. 83, *MS, ST*). However, at a higher level, these double stamens from the one sepal bundle, unite. The alternating bundles represent the lateral sepal-gland-carpellary bundles. The semi-circular truncate gland is a three-lobed structure, united basally, but the anterior lobe is absent (Fig. 86, *G*). At the base of each gland, two bundles from adjacent lateral sepal bundles send strands which ramify slightly toward the fleshy, glandular lobes (Fig. 85, *GT*). The remaining receptacular traces plus traces for the suppressed anterior lobe of the semi-annular gland constitute the gynoecial cylinder of sixteen to twenty bundles in the gynophore. The dorsal with its two branches, the two ventrals and numerous laterals ascend through the ovary wall. All laterals, but a pair between the dorsal and ventral carpellaries on each side fade out above the tip of the ovary loculus (Fig. 89). The style is fusiform, the stigma partly terminal with a pollen-collecting device (Fig. 90, *SM*). Each broad filament contains the fused single stamen trace, which terminates in the broad connective. The numerous ascending ovules are imbricated in two to four series, vascularized by traces from the ventrals (Fig. 88, *O*). The strands of the ventral, the dorsal and its branches with two pairs of lateral strands continue upward through the carpel.

Embothrium characteristics are: the conspicuous furrowed inflorescence axis composed of some lengthy peduncles conjoined to the cortex of the rachis, except for the tip; the numerous ascending ovules in two or four series; the three-lobed semicircular gland; basally open carpel, triporate pollen; double stamens, and a pollen-collecting device.

The various species studied revealed the general pattern of vascularization as found in *E. mucronatum*. Some morphological features vary. In *E. grandiflorum*, the rachis eustele is composed of an almost continuous ring of vascular tissue, but capped by a concentric ring of heavy sclerenchyma fibers. The peduncles are exceedingly long, often two or three inches conjoined to the rachis, but free about one-half inch at the tip where twin pedicels arise. The inflorescence is a large umbel based by compact leaves. In *E. coccineum*, the peduncles are very short, the flowers few and clustered, leaves more numerous than flowers.

Lomatia dentata R. Br.— Four American species of *Lomatia* were examined. Anatomically the origin and course of the vascular skeleton of the branch systems and the zygomorphic flowers are similar.

The peduncle with its deciduous bract originates from the rachis by means of the usual branch traces, and in turn forms two pedicellate steles (Figs. 95, *PD*; 96, P_1, P_2). The bundles at the base of the oblique receptacle abruptly and almost simultaneously dissociate into the necessary bundles. Four calycine bundles and their respective staminal bundles in the same radius, and eight lateral sepal bundles are prominent in the receptacle (Fig. 97, *MS, LS*). All lateral sepal bundles separate into the marginal sepal bundles and the gland bundles (Fig. 99, *GB*). Each of three broad, truncate glands is vascularized by two bundles at the base. These bundles send traces com-

FIGS. 93-109 — *Lomatia dentata* R. Br.   Fig. 93. Geminate floral buds showing protrusion of ovary and style between calyx lobes. × 2.   Fig. 94. A single flower (calyx removed) depicting long gynophore, four glands, ovary and style. × 2.   Fig. 95. Peduncle with sclerenchyma ring enclosing vascular cylinder. × 40.   Fig. 96. Two pedicellate eusteles formed from peduncle. × 40. Fig. 97. C.s. of slightly oblique receptcle, showing distribution of various bundles. × 20.   Fig. 98. Receptacle at higher level. × 15.   Fig. 99. Calycine traces and vascular bundles to glands. × 15. Fig. 100. Three glands and their vasculature. × 18.   Fig. 101. Ramifications of vascular tissue within the three glands. × 18.   Fig. 102. C.s. of ovary with several pendulous ovules; ovular traces from ventral strands. × 30.   Fig. 103. Base of gynophore, three free lobes of glands. × 30. Fig. 104. Anthers sessile to calyx, broad connectives. × 30.   Figs. 105-109. C.s. of stigma at various levels. × 40.

posed of elongated phloem cells through the gland toward the secretory tissue (Figs. 100, 101).   The remaining pair of lateral sepal bundles, the branches of stamen bundles and a few isolated bundles from the receptacle enter the long gynophore.   The vascular ring of the latter is composed of about twelve bundles: one strong dorsal, two ventrals and branches of these (Figs. 102, *VC, DC*).   The laterals in their upward movement gradually unite in various ways, the manner of which was most difficult to determine.   At a higher level, six bundles were observed; still higher in the style only three were seen

which seem to represent fused bundles, however, dissociating in the oblique disc with a stigmatic center (Fig. 107, *DC*). At anthesis, the style protrudes through the slit on the ventral side of the calyx (Fig. 93).   The many anatropous ovules, laterally attached below the middle of the loculus (Fig. 102, *O*), are supplied by the branches from the ventral carpellary strands.   Each stamen vascularized by a single bundle is adnate to its adjacent sepal by means of a broad filament and connective (Fig. 104, *N*).   The anthers are free only at the tip. The connective is non-protruding.   The pollen is triporate.

The style is oblique at the tip, with a laterally expanded stigma and with a pollen-collecting device (Figs. 106, 107).

The genus *Lomatia* is noted for its three-lobed heavily vascularized semi-annular gland, almost sessile anthers, stipitate ovary, numerous anatropous ovules laterally attached and a pollen-collecting device.

Discussion[3]

From the foregoing observations, it is apparent that there is a more or less uniform plan in the vascularization of the American species of Grevilleae and Embothrieae. In general it duplicates, except in a few minor instances, the Australian taxa studied in the same tribes.

INFLORESCENCES — The morphological and anatomical investigations of the American species emphasize all the more the evolutionary trends suggested for the Australian species of the Proteaceae. There is less variation in the types of inflorescences, the majority constituting axillary or terminal racemes, either dense or lax. These species emphasize the conclusion that the primitive type was a panicle. The more compact thyrse is found in *Embothrium*. Secondary branches may be short or elongated peduncles. Each bears geminate flowers in all species. Single or sessile flowers were absent in these American species examined since all belong to the sub-family Grevilloideae. Compression resulting in the reduction or loss of branching systems of the panicle is evident. In the reduction of the axes of the peduncle, the secondary branch systems first may have proximated and paralleled the rachis and still further compression led to the conjoining of the peduncles to the main axis. The result is the furrowed rachis of the inflorescence of *Embothrium* and *Roupala*. In *E. grandiflorum*, the peduncles are connate for some length; in *E. mucronatum*, they are shorter, while in *E. coccineum*, there is still further reduction in length. These peduncles become free from the main axis for various lengths, in the area where their twin pedicels are formed.

Phylogenetically the primitive paniculate type has undergone reduction throughout the family, but to a lesser degree among American species. The conjoined peduncles of *Embothrium* and *Roupala* is a step in compression not found in the Australian forms of Grevilloideae examined. The inflorescences of American genera are specialized also due to (1) the shortening of secondary branch systems, (2) abortion and loss of bracteoles, (3) the gradual reduction of vascular tissue at the base and within the glands.

THE CALYX — In all species examined, the median sepal bundle is clearly defined in the base of the receptacle. Commissural bundles are evident in *Panopsis*, *Guevina* and *Euplassa*. In *Embothrium*, the commissural or lateral sepal bundles have already dissociated and appear as separate identities in the base of the receptacle. So ultimately each perianth segment is vascularized by three foliar traces. This emphasizes the conclusion that the proteaceous perianth is a definite calyx. Three foliar bundles are characteristic of primitive foliar organs (Eames & MacDaniels, 1947). Phylogenetically, the three-traced sepal derived from the receptacular stele is considered the most primitive. Due to the shortening of the floral axis and the internodes in the receptacle, there has been a closer proximity of adjacent lateral bundles producing a common bundle, the commissural, and bringing it into the plane of petal traces (Kavaljian, 1952). Kausik (1938) concludes " the alternating single strands supplying the margins of adjacent perianth lobes are really petal traces, the courses of which have become altered on account of complete disappearance of a whorl of petals; these traces, therefore, enter the lobes of the only surviving envelope ", which in this case is the sepal. However, if petals are produced, reduction may cause the fusion of the lateral sepal and petal trace, as will be discussed later. Single-traced sepals or sepals in which the lateral or marginal traces emerged from the median sepal bundle in the lamina were not observed in these genera.

THE STAMENS — All the epiphyllous stamens are perfect. The broad filament is adnate to the sepal; anthers sub-sessile,

3. This paper does not discuss the various conclusions of other writers whose works may bear on the ideas expressed herein. This was done in detail in the author's first paper.

free for a short distance above the base of the lamina. Apiculate connectives characterize *Euplassa, Roupala, Panopsis, Guevina* and *Embothrium*.

In the majority of species a single bundle traverses a stamen. In *Grevillea robusta*, Kausik (1941) reported double stamens. This author found the same situation. Double stamen bundles in the receptacle appear in *Embothrium mucronatum*, but before the filaments are separated from the calyx, they became fused. One pair in this species persisted into the connective of the anthers. The two-traced stamen represents the primitive condition, while a single trace denotes a fused one. Adnation of traces is another evidence of compression, as is found in the union of midrib bundles of the sepals and the epiphyllous stamens. Triporate pollen was present in all species examined.

THE GLANDS — The most discussed organ of the Proteaceae is the gland, disc or scale. All three types appear among the American species. Diverse morphological theories have been advanced: (1) " a non-vascular organ in connection with secretion of nectar " (Rao, 1957); (2) " an adventitious accessory development of the torus " (Brough, 1933); " a reduced corolla " (Kausik, 1941). The American species present a reduction series in the number of glands in the whorl: four free lobes in *Euplassa*, a disc or cup with a four-lobed rim in *Panopsis*, three basally fused lobes with the anterior one suppressed in *Grevillea*, three free scales in *Roupala*, three fleshy glands in *Embothrium* and *Lomatia*; two lobes only in *Guevina*. No flowers without glands were evident. Hence the species exhibit a reduction from four glands to three, to two.

The vascular supply for these glands originates from the commissural bundles directly after their radial division, or from the lateral sepal bundles directly in the base of the receptacle. In the actinomorphic flowers, the glands are symmetrical and their vascular tissue is derived from lateral sepal bundles all around the stele. In the zygomorphic ones, it is derived from those bundles in close proximity to the glands. *Lomatia* and *Embothrium* possess strong bundles, the branches of which pass into the gland. In *Lomatia* the phloem

cells produce a more or less fan or brush-shaped arrengement within the gland. In *Panopsis* and *Guevina*, traces penetrate the gland for a short distance only. In *Euplassa*, the vascular tissue is weak, possessing only a few elements. Hence there is a reduction of vascular tissue among the American species.

The four distinct lobes of the glandular ring indicate the presence of a tetramerous whorl, which is complete in the actinomorphic flowers. Where zygomorphy is dominant, the semi-annular gland position indicates that the abaxial or anterior lobe or lobes are obliterated due to suppression. These whorls are either tri- or dimerous.

The Proteaceae show a phylogenetic reduction of a petal whorl, which has become transformed in its morphology so as to affect a secondary role as a nectariferous organ. The glands are homologous to petals as evidenced by (1) the origin and behaviour of the vascular bundles and traces in the petal sectors of the receptacle; (2) their alterni-sepalous position; (3) the tetramerous plan of the primitive forms. Reduction has played a role in this whorl.

THE GYNOECIUM — The gynoecium consists of a solitary carpel, occupying a terminal position on the axis. Among the species examined there is a remarkable degree of uniformity in the vascular system, but the number of ovules per carpel, the number of accessory branches of the dorsal and particularly the lateral carpellary bundles vary. The pollen-collecting devices vary in shape or may be absent.

The ovary of the monocarpel may be sessile (*Roupala, Panopsis*) or borne on a gynophore (*Euplassa, Guevina, Embothrium*). It possesses a single loculus bearing two orthotropous pendulous ovules (*Roupala, Panopsis, Guevina, Euplassa*); four in *Lomatia*; which are laterally attached below the middle; many in *Embothrium* where two to four series of ascending and imbricated ovules develop.

Since the conduplicate carpel with laminate placentation is now considered a primitive type of gynoecium, the proteaceous carpel of American as well as Australian species represents a modified conduplicate type with open ventral suture in the base

of the ovary, often near the tip. The placentation is sub-marginal. *Embothrium* and *Lomatia* may suggest remnants of the laminal placentation. Thus it is postulated that the carpel of the Proteaceae is a primitive one. In the Grevilloideae the adaxial surfaces of the monocarpels in the twin flowers face each other.

Fundamentally the monocarpel is a three or five-traced organ, in which the laterals may vary in number from one to nine traces on each side of the ventral margins. This increase results from amplification of the dorsals to three, and of the basic laterals. This behavior is characteristic of both tribes.

The carpellary vascular system arises generally from the triple-fused lateral sepal-gland bundles and from isolated bundles in the vascular stele of the receptacle. The ovules are vascularized by branches of one of the two ventral bundles.

The *Embothrieae* because of their many ovules are considered a primitive sub-tribe; the *Grevilloideae* with one or two ovules generally a step toward specialization. There were no abortive ovules nor evidence of vestigial traces. The reduction in the number of ovules seems to have taken place from the base upward to the tip of the loculus. This reduction is closely correlated with the number of flowers in the whole inflorescence: a fewer number among the loose racemes; greater number with compact or denser types (*Embothrieae*). The majority of ovules are of the orthotropous type which are considered more advanced. The great number of bundles or branches of vascular tissue in the ovary, and the partially opened carpel may be an adaptation for bird pollination which is so common in this family.

Thus the carpels of the American Proteaceae, though exhibiting some primitive features, represent highly specialized ones. The conduplicate carpel with sub-marginal ovules, the reduction of the number of ovules, the general development of orthotropous ovules, the partial or complete closure of the ventral surfaces in the stylar and ovular regions indicate advancement. Hence carpels of the Proteaceae are specialized, brought about by reduction of structures and their vasculature.

ZYGOMORPHY — Zygomorphy is generally associated with racemes (Stebbins, 1949). In the irregular flowers, zygomorphy has manifested itself in the oblique receptacle, in the glands which have become modified by suppression of some lobes in the anterior position and the consequent fusion of surviving lobes. The foot of the ovary is usually welded to one side of the perianth. The base of the ovary is generally opened on its adaxial side, and the gland is usually deepest in this vicinity. The carpel develops more or less in the position of the suppressed lobe. When the gland and the ovules mature, the lengthened style causes a separation of two sepals in an anterior-posterior plane to allow for the protrusion of the style. The stigmatic tip is firmly enclosed in the connate sepal tips till anthesis. This slit-like opening affords an easy method for honey-eaters and sun birds to reach the abundant nectar. " The more extreme the zygomorphy, the greater is the number of floral parts affected by irregularities and more marked are differences between them ". (McLean & Ivemey-Cook, 1956.)

Conclusions

The comparative anatomical studies of American species reveal added evidence that the Proteaceae are highly specialized and exhibit a series in flower modification which exemplifies the evolutionary tendencies within the family. The Grevilleae in some features are more advanced than the Embothrieae. The flowers ultimately terminate branches of a secondary branch system of a raceme derived from an ancestral panicle.

Specialization has been manifested along various lines: the shortening of secondary branches except in *Embothrium*, the disappearance of some bracts and bracteoles, the modification of a petal whorl from membraneous organs to fleshy free glands, to a semi-annular ring; the reduction of the number of organs in the petal whorl; the cohesion of vascular strands; the adnation of floral whorls, the decrease in number of ovules, the presence of pendulous orthotropous ovules, and the presence of pollen-collecting devices. All

these indicate specialization through reduction.

This complexity and evolutionary reduction has resulted from a suppression of nodes and internodes, closer aggregation of peduncles of a panicle (*Embothrium*) and also their shortening. Reduction parallels specialization (Stebbins, 1949). Anatomical investigations disclose the fact that the ancestral flower was dichlamydeous and reduction has been the chief factor for apetaly.

Summary

The American species of the Proteaceae belong to two tribes only, the Grevilleae and the Embothrieae. Twenty-three species of seven genera were examined. The vascular anatomy reveals a degree of uniformity. The geminate flowers are highly specialized in an advanced state of reduction. Each flower is composed of a tetramerous calyx with epiphyllous stamens. The petal whorl, composed of glands, discs or scales, basically is tetramerous, but due to loss, tri- and dimerous corollas exist. The members of this whorl are alterni-sepalous in position, and their vascular supply emanates from the lateral sepal bundles in the receptacle. The American and the Australian species of these tribes lead to the conclusion that the proteaceous ancestor was dichlamydeous. The family probably is a primitive one, but highly specialized phylogenetically. Its systematic relationship will be considered when African taxa are studied.

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IN VITRO CULTURE OF OVARIES OF *IBERIS AMARA* L.

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Introduction

The experimental approach to the study of flowers and fruits, introduced by La Rue (1942) and Nitsch (1949, 1951), has been lately adopted by several workers. Rédei & Rédei (1955) used it to obtain viable embryos of *Triticum spelta*. They cultured ovaries from the middle portion of the pollinated spikes and noted that embryos differentiated only if the paleae were left attached to the ovaries. Nevertheless, the endosperm was arrested and the caryopses showed only a slight increase in size. The embryos were excised and transplanted on a fresh medium.

De Capite (1955) grew the ovaries of a cross between *Fragaria chiloensis* and *F. virginica* in artificial media but the fruits failed to reach the size attained *in vivo*. In *Pisum sativum* also the fruits obtained were smaller than those in nature.

Anantaswamy Rau (1956) investigated the influence of colchicine on the artificially reared ovaries of *Phlox*. This induced some interesting abnormalities in early embryogeny and endosperm.

At the University of Delhi attempts have been made to culture the ovaries of *Linaria* (Sachar & Baldev, 1958), *Tropaeolum* (Sachar & Kanta, 1958), *Althaea* (Chopra, 1958), and an apomictic species of *Aerva* (Murgai, 1959). After trying a large number of chemicals Sachar & Baldev (1958) found yeast extract to be the most suitable supplement for growing the ovaries of *Linaria maroccana*. The apomictic embryos of *Aerva* also grew best when the ovaries were grown on a medium containing yeast extract (Murgai, 1959). Chopra (1958) was able to produce parthenocarpic fruits in *Althaea* by incorporating IBA in the nutrient medium.

The present paper is a continuation of the work reported earlier with the ovaries of *Iberis amara* (Maheshwari & Lal, 1958)

Material and Methods

Iberis amara is a winter annual. In each inflorescence 5-8 flower buds, usually belonging to one ' whorl ', open in the morning. The stigma of the freshly opened flower has a greenish tinge. Towards the afternoon and certainly by the evening, when the flowers have been pollinated, it turns a bright yellow. The pollen tubes grow rapidly and next morning the ovaries show fertilized ovules. Meanwhile the stigma changes to a crimson or a dark brown shade. The growth of pollen tubes was traced by acetocarmine squashes of the stigma and style and the formation of zygotes in the material gathered one day after pollination was confirmed by sectioning. In order to obtain material of a uniform age those flowers in which the stigma had turned yellow were marked by a red ink dot on a petal. They were gathered the next day for inoculation when the embryo sac invariably showed the presence of a zygote or a few-celled proembryo and free endosperm nuclei. The average size of the ovary at this stage was $3 \cdot 7 \times 2 \cdot 0$ mm.

The nutrient medium was prepared according to the formula given by Nitsch (1951) with the addition of 5 ml/l of White's vitamin solution modified by making it $2 \cdot 5$ times concentrated and adding calcium pantothenate ($0 \cdot 25$ mg/l). It is designated as NBV in the text.

Flowers were brought from the garden in a beaker of water. The pedicels were cut to similar lengths and the stamens were removed. The calyx and corolla were retained in all except when their role in fruit formation was to be studied. The material was disinfected by treating

with a 10 per cent clear solution of calcium hypochlorite for 20 minutes. It was then twice rinsed with sterile distilled water. Before planting in the medium, the bleached end of the pedicel was trimmed with a sterile scalpel. Usually 48, sometimes 96, flowers were inoculated in each medium.

Records of measurements of ovaries in the field were made daily from the day of anthesis until maturity of the fruit. The length was measured from the base of ovary to the tip of stigma while the breadth was measured along the greatest diameter, somewhere in the middle of the fruit. The sizes given in the text represent averages of 6 ovaries. The age of the ovaries or fruits is given in days after pollination.

The cultured material was fixed in formalin-acetic-alcohol at intervals of one week. Ovaries of corresponding ages growing in the field were also fixed to enable comparisons between the *in vitro* and the *in vivo* growth.

Material of some selected treatments was imbedded in paraffin and sectioned and stained in the usual way.

Observations

GROWTH IN VIVO — The growth of the ovary is conspicuously stimulated after fertilization and fruits collected a week after pollination measure twice their initial size (Figs. 1, 2). By this time the embryo reaches the globular stage and cell formation begins in the endosperm. The latter process comes to completion in a couple of days and with it there is another steep increase in the volume of fruit. During this period the fruit wall hardens, the veins become prominent and

the corolla is shed followed by the calyx. The maximum size is attained in 12-15 days after pollination (Fig. 3) when the fruits measure approximately 9.1×7.6 mm. The embryo continues to grow and attains maturity only after another week. Figures 22-25 show the development of embryo *in vivo*.

GROWTH IN VITRO — Flowers planted one day after pollination in the basic medium (NBV) developed into fruits which were normal in most respects. As with ovaries growing in nature, there was an appreciable increase in size soon after fertilization, after which the rate of growth levelled off. When the embryo was progressing from the globular to the heart-shaped stage, about 6-9 days after pollination, there was another peak in fruit growth. Subsequently the fruits grew but not as much as in nature and after about two weeks growth ceased. The cut end of the pedicel swelled and showed a slight callusing. However, the embryo continued to enlarge and reached maturity a week after perceptible fruit growth had ceased.

Although the ovaries reared in the basic medium (Fig. 7, *see* also Table 1) resembled natural fruits, they were pale in colour and the veins were not prominent. The fruits also failed to dehisce in the test tube.

A few experiments were conducted to ascertain how far the constituents of the basic medium could be cut down without hampering the growth of the ovaries.

In the simplest experiment two sets were maintained in water: one with individual flowers and the other with whole inflorescences. The growth of the former was poor and ceased after one week, the size attained being only 4.6×2.5 mm

→

FIGS. 1-13 — Figs. 1-3. Growth of fruits *in vivo*; Figs. 4-13. Growth of fruits *in vitro*. Fig. 1. Ovaries excised one day after pollination. × 2. Fig. 2. One-week old fruit. × 2.5. Fig. 3. Two-week old fruit. × 3. Fig. 4. Fruit grown in distilled water; one week old. × 2. Fig. 5. A two-week old fruit grown in 5 per cent sucrose. × 3. Fig. 6. Fruit grown in Nitsch's basic (NB) medium (two-week old). × 2.5. Fig. 7. Two weeks' growth in Nitsch's basic + vitamins (NBV) medium. × 3. Fig. 8. Another fruit from NBV + IAA 5 mg/l medium (two-week old). × 2.5. Fig. 9. A two-week old fruit from NBV + kinetin (0·5 mg/l) medium. × 3. Fig. 10. Fruits grown in NBV + kinetin 0·1 mg/l + IAA 5 mg/l. × 3. Fig. 11. Fruits from NBV + kinetin 0·5 mg/l + IAA 5 mg/l. × 3. Fig. 12. A fruit from NBV + kinetin 0·6 mg/l + IAA 5 mg/l × 3. Fig. 13. Fruits from NBV + kinetin 0·5 mg/l + IAA 10 mg/l. Note the complete absence of callus formation. × 3.
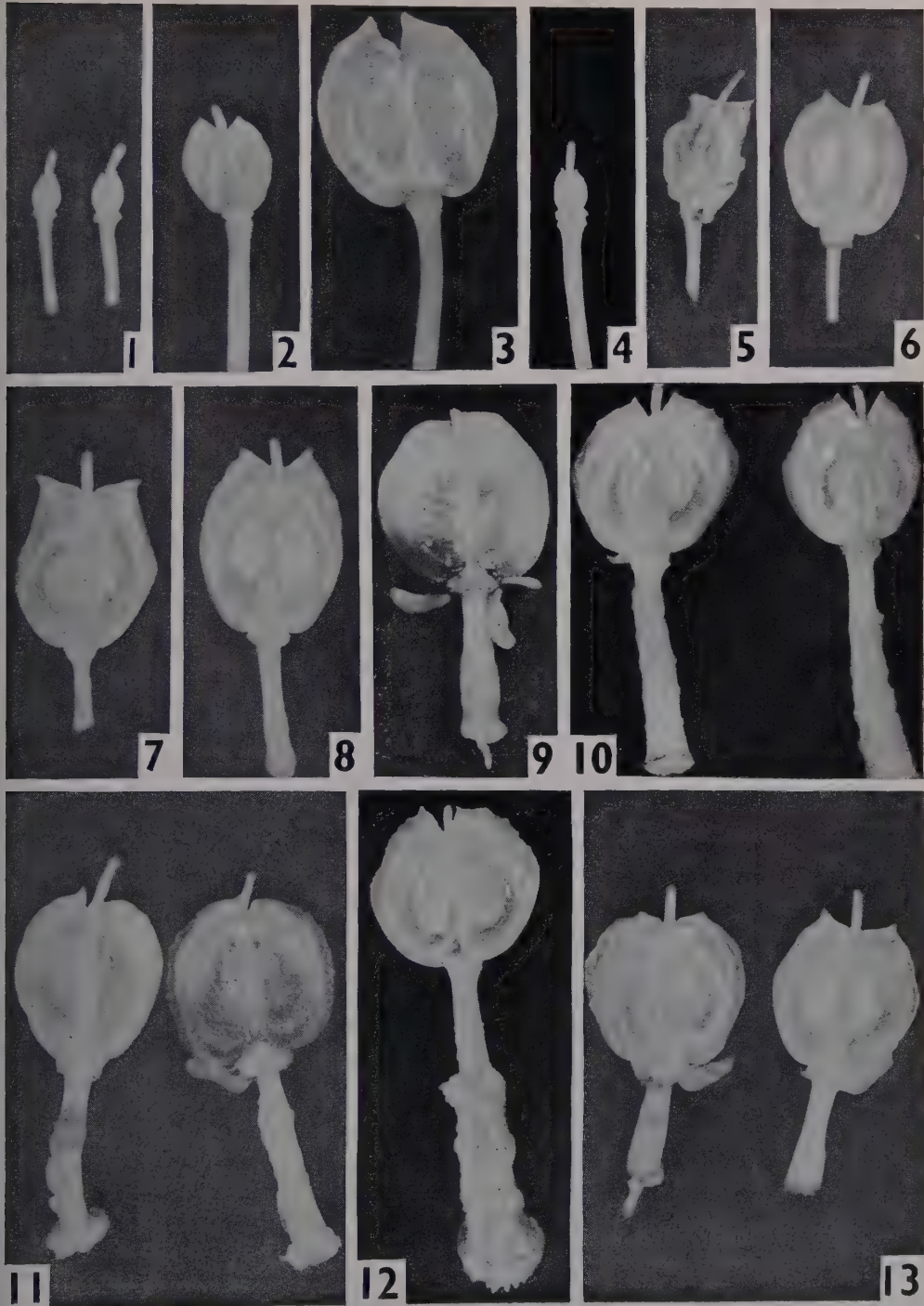
TABLE 1 — GROWTH OF THE OVARIES *IN VIVO* AND *IN VITRO* (NBV)

(Initial size : 3·7 mm long × 2 mm broad)

	FIRST WEEK	SECOND WEEK	THIRD WEEK
In vivo			
Ovary	7 × 5 mm	9·1 × 7·6 mm	9·1 × 7·6 mm
Embryo	Globular	Cotyledons differentiated	Full sized cotyledons
Endosperm	Free nuclear	Cellular	Cellular
In vitro			
Ovary	6·8 × 6 mm	9·1 × 6·8 mm	9·1 × 7·5 mm
Embryo	Globular	Heart-shaped	Elongation of cotyledons
Endosperm	Cellular	Cellular	Cellular

(Fig. 4). As may be expected, the fruits were larger (5·4 × 3·0 mm) when the whole inflorescence was taken. Further, no embryo or endosperm was formed in isolated flowers while in the inflorescence the embryo grew up to the globular stage.

In sucrose (5 per cent) + agar (0·8 per cent) the ovaries grew for two weeks and enlarged to almost twice their initial size. However, the fruits were cartilaginous and showed uneven margins (Fig. 5). The seeds lacked both endosperm and embryo.

If minerals were added to the above medium, there was a marked improvement in growth. The ultimate size of the fruit and the embryo was, however, a little smaller than that in nature (Figs. 6, 26).

Besides nutrients, the calyx and corolla also play an important role in the growth of the ovaries. In one set of experiments, the calyx and the corolla were removed in flowers excised 1, 2, 4, 6, 8 and 12 days after pollination and the ovaries planted on the basic medium. The ovaries inoculated 1, 2 and 4 days after pollination failed to grow. Even those taken 6 days after pollination attained only a little more than half the size of normal fruits. However, there was no significant difference in the size of the cultured fruits if calyx and corolla were clipped off 8 or 12 days after pollination. Later two sets were run in which either the calyx or the corolla was removed. From these it seemed clear

that it is the calyx and not the corolla which to a large extent controls the growth of the ovary. In the field, however, the removal of the calyx from a few flowers in the inflorescence (irrespective of the stage of development) did not affect the growth of ovaries. Presumably the contribution of calyx is nutritive rather than stimulatory, analogous to that of the leaves in inducing fruit set in tomato (*see* Leopold & Scott, 1952).

The addition of IAA (5 mg/l) to the basic medium improved ovary growth, the average size attained being 10·2 × 8·4 mm (Fig. 8). The embryos and endosperms dissected at weekly intervals also showed normal growth. Kinetin added to the basic medium in concentrations ranging from 0·1 to 0·6 mg/l gave very good results in the initial stages but subsequent growth slowed down and even the largest fruits measured only 8·4 × 7 mm (Fig. 9). The addition of IAA 5 mg/l did not enhance the effect of kinetin.

Often there was active callus formation on the pedicel after the maturity of the fruit (Figs. 10-12). This could be inhibited or greatly reduced without affecting fruit growth by increasing the concentration of IAA from 5 mg/l to 10 mg/l (Fig. 13).

Since in the initial stages kinetin and IAA appeared to support an optimal growth of fruits, we used maleic hydrazide to see if the effect could be reversed. In one such experiment the concentration of kinetin was 0·5 mg/l and of IAA

FIGS. 14-21 — Fruit growth *in vitro*. Fig. 14. Fruits from NBV + kinetin 0·5 mg/l + 2, 4-D 2 mg/l. The fruits are irregular in outline and the seeds in them are abortive. × 3. Figs. 15-18. Fruits grown in the above medium supplemented with 1, 5, 50 and 100 mg/l of MH respectively. Note profuse callusing on the stalk of the fruit in Fig. 15. Figs. 15, 16, ×2·5; Figs. 17, 18. × 3. Figs. 19. Fruit showing callus formed at the junction of pedicel and fruit proper in NBV + colchicine medium. × 5·5. Fig. 20. Callus formed in patches distributed all over the stalk in NBV + kinetin (0·5 mg/l) + IAA (5 mg/l) medium. × 2·5. Fig. 21. Callus formation at the cut end of the fruit stalk in NBV + kinetin (0·5 mg/l) + adenine (10 mg/l). × 2·5.

5·0 mg/l while that of MH was 1, 5, 50 and 100 mg/l. In the lower concentration of MH (e.g. 1 mg/l) the fruits measured 9·0 × 7·7 mm in two weeks and were thus larger than in kinetin media. At the same time the seeds contained mature embryos. The rate of development remained the same as in the basic medium, i.e. globular embryos were formed in 8 days and mature embryos in 21 days, but there was some proliferation of the radicle and the cotyledons (Figs. 27, 28).

When 5 mg/l of MH was added to the medium, the growth of the pericarp was still almost normal; in a week's time the size of the ovary doubled and after two weeks it matched with fruits obtained with 1 mg/l MH (Fig. 16). However, the development of the embryo was strikingly retarded. In one week old cultures the ovules showed a young proembryo and free nuclear endosperm (Fig. 29). After

12 days in culture, the endosperm and embryo showed signs of degeneration and after 21 days, when mature embryos were expected, only malformed octants (Figs. 30, 31) and disorganized endosperm were observed.

When 50 mg/l or 100 mg/l MH was used, the growth of the ovaries was still poor (Figs. 17, 18) and the fruits did not exceed 6·7 × 4·5 mm. There was no trace of an embryo and the endosperm was represented only by the degenerating remnants of the nuclei.

With kinetin (0·5 mg/l) and 2,4-D (2 mg/l) the ovaries remained smaller (7·4 × 6·2 mm) than those in the basic medium alone and the edges of the pericarp were uneven and distorted (Fig. 14). The more important changes included lack of development of endosperm, aborted seeds, and irregular thickenings on the veins of the petals. On sectioning, the latter were found to comprise masses of densely cytoplasmic, parenchymatous cells. These always developed adjacent to the phloem.

The addition of adenine (5, 10 mg/l) to the kinetin + IAA medium had no marked effect on fruit growth.

The growth of the fruits was considerably retarded by colchicine (50, 100, and 200 mg/l). In two weeks the ovaries did not grow beyond 7 × 6 mm. With increasing concentrations, the tendency for ovular abortion became pronounced. The ovules which did not abort showed small embryos and a poorly developed cellular endosperm. At 200 mg/l the endosperm showed the formation of nodules each containing 3-5 nuclei.

FORMATION OF CALLUS AND ROOTING — A common feature in a number of cultures was the formation of callus on the pedicel. In adenine media it was restricted to the cut end of the pedicel (Fig. 21) while with colchicine it developed at the junction of the ovary and the pedicel (Fig. 19). The most striking development was recorded in kinetin media (Figs. 12, 20), particularly in kinetin (0·6 mg/l) + IAA (5 mg/l) and in kinetin (0·5 mg/l) + IAA (5 mg/l) + MH (1 mg/l) (Fig. 15). It extended to the entire length of the pedicel and was more or less uniformly thick all round.
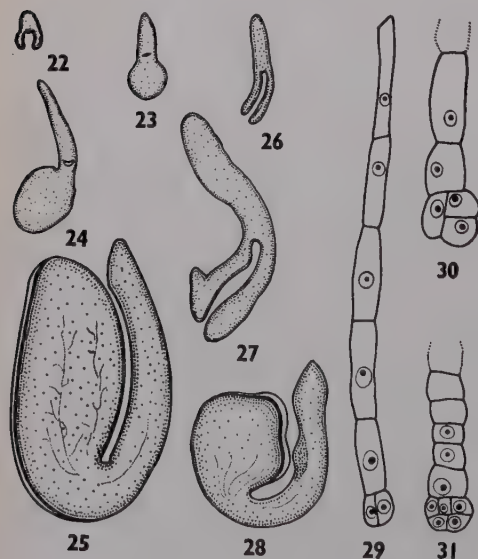
FIGS. 22-31 — Median longitudinal sections of embryos produced *in vivo* and *in vitro*. Figs. 22-25. Embryos formed in 10, 12, 15 and 21-day old fruits *in vivo* respectively. × 20. Fig. 26. A three-week old embryo formed in fruits grown in sugar + minerals. × 20. Figs. 27, 28. Twenty one day old embryos formed in NBV + kinetin (0·5 mg/l) + IAA (5 mg/l) + MH (1 mg/l). ×20. Figs. 29-31. Six, 12 and 13-day old embryos formed in the medium containing MH 5 mg/l. × 475.

Rooting occurred sporadically in the plain mineral medium, in NBV, in NBV + IAA and in kinetin + IAA media.

Summary and Conclusion

Flowers of *Iberis amara* L. sown one day after pollination in Nitsch's basic medium supplemented with vitamins yielded normal and healthy fruits which matched those in nature. However, this was not possible if the calyx was excluded before planting. Fairly good growth of ovaries was also possible by merely supplying sugar and minerals but the embryos were smaller than those in nature. The addition of IAA to the basic medium had a slightly stimulative effect on fruit growth and occasionally fruits slightly larger than those set in nature were formed. Kinetin (0·5 mg/l) did not very much modify fruit growth but along with IAA (5 mg/l) it caused an overgrowth of callus on the pedicel. Keeping the concentration of kinetin at 0·5 mg/l, if the level of IAA was raised from 5 mg/l to 10 mg/l, the development of callus was almost suppressed. Lower concentrations of maleic hydrazide (added to the kinetin + IAA medium) showed no inhibition of pericarp or seed, while higher concentrations were deleterious for seed formation. 2,4-D (2 mg/l) (used in combination with kinetin) caused an inhibition of seed development. Colchicine was inhibitory for fruit development and higher concentrations increased the tendency for ovular abortion. Adenine (used with kinetin + IAA) had no marked effect on fruit or seed formation, except the induction of callus at the cut end of pedicel.

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SOME EMBRYOLOGICAL ASPECTS OF *EUPHORBIA DULCIS* L.

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In 1901 Hegelmaier recorded an interesting case of polyembryony in *Euphorbia dulcis* and noted that in this plant some embryos originate from the nucellus and the others from the egg apparatus. Two years later he published (Hegelmaier, 1903) a more detailed and illustrated account confirming his previous observations. However, he failed to ascertain whether pollination or fertilization occurred, and suspected *E. dulcis* to be apogamic and parthenogenetic.

Winkler (1908; quoted in Carano, 1926) drew attention to the possibility of the occurrence of polyembryony in the absence of pollination, but concluded that further investigation was necessary. According to Ernst (1918; quoted in Carano, 1926) stimulation by pollen was essential for the production of fruit, seed and embryo.

Carano (1925, 1926) noted the Fritillaria type of embryo sac in *E. dulcis* but so far this type has not been reported in any other species of *Euphorbia*. In 1946, Maheshwari remarked: " It would be interesting to know the behaviour of *E. dulcis* in places other than Italy ". The object of taking up this reinvestigation was, therefore, twofold. First, to determine if *E. dulcis* is an apomict and whether the stimulus of pollination and fertilization is necessary for the production of nucellar embryos. Secondly, to check Carano's observations in the light of Maheshwari's suggestion.

Material and Methods

This investigation is based on the material of *Euphorbia dulcis* obtained by Professor P. Maheshwari through the courtesy of Professor O. Hagerup, Botanical Museum, Copenhagen, Denmark. The first lot of imbedded material (labelled June 1953) proved to be inadequate but Professor Hagerup was kind enough to send another instalment (May, 1954). Later he sent further material preserved in formalin-acetic-alcohol which had been collected during June, 1955; May, 1957; and May, 1959.

Depending upon the age and size of the material sections were cut 5-15 microns thick. The safranin and fast green combination gave excellent results.

Observations

INFLORESCENCE AND FLOWER — The inflorescence generally shows considerable reduction in the number of male flowers and it seems to be a characteristic feature of this species (*see* also Hegelmaier, 1901, 1903; Carano, 1925, 1926). However, there is a single female (Fig. 55) flower which is centrally placed in the inflorescence. The ovary is covered with many unicellular hairs. Due to the active growth of the pedicel, at the time of anthesis, the pistil usually extends beyond the involucre (Fig. 61).

FIGS. 1-9 — Megasporogenesis. Fig. 1. Megaspore mother cell. × 1030. Figs. 2-4. Two and 4-nucleate coenomegaspores. × 1030. Figs. 5-7. Megaspore nuclei showing 2+2 and 1+3 arrangements. × 1030. Fig. 8. The three chalazal nuclei are in the process of fusion. × 1030. Fig. 9. Embryo sac with 1+3 arrangement showing division of nuclei. × 1030.
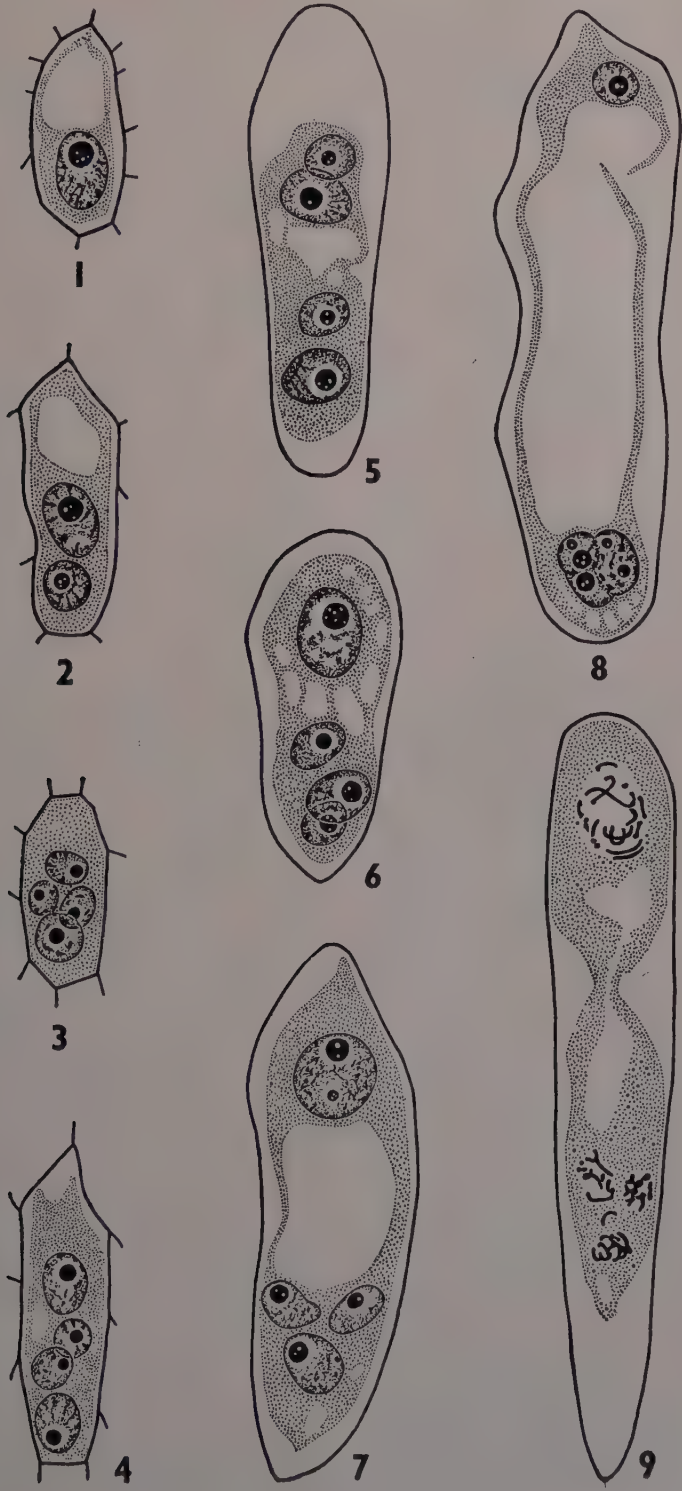
FIGS. 10-17 — Female Gametophyte. Fig. 10. Secondary 2-nucleate embryo sac. × 720. Fig. 11. Embryo sac showing one micropylar and two chalazal nuclei. × 720. Figs. 12, 13. Secondary 4-nucleate embryo sacs; in Fig. 12 the micropylar nuclei are appreciably larger. × 720. Fig. 14. Later stage; the chalazal nuclei have divided earlier than the micropylar. × 720. Fig. 15. All the four nuclei are in division. × 720. Fig. 16. Eight-nucleate embryo sac with organized antipodal cells. × 720. Fig. 17. Mature embryo sac (Fritillaria type). × 720.

MICROSPOROGENESIS AND MALE GAMETOPHYTE — For want of adequate material a detailed investigation of the development of male gametophyte could not be carried out. Like other species of *Euphorbia*, the anther shows four wall layers — the epidermis, endothecium, middle layer, and the uni- or binucleate tapetum. The middle layer and tapetum generally degenerate at the uni-nucleate stage of the pollen grains.

The development of microspores proceeds normally and tetrahedral, isobilateral and decussate tetrads are formed. Some of the microspores degenerate while still within the tetrad (*see* also Carano, 1926). Widespread disorganization of pollen grains starts at the uni-nucleate stage but some may survive and attain the 2-celled condition. However, these also degenerate soon after and, therefore, fertile pollen grains fail to develop.

MEGASPORANGIUM, MEGASPOROGENESIS AND FEMALE GAMETOPHYTE — As in other species of *Euphorbia*, the trilocular ovary contains in each locule a single, bitegminal and crassinucellar ovule on an axile placenta. The elongated cells of the obturator sometimes contain abundant starch.

According to Carano (1926) the archesporial cell cuts off a parietal cell which undergoes repeated divisions. My preparations also showed a megaspore mother cell covered by two or three layers of parietal tissue. The nucleus of the megaspore mother cell is usually situated in its lower part (Fig. 1). Of the two nuclei produced as a result of meiosis I, one is comparatively smaller (Fig. 2). The second reduction division leads to the formation of four free megaspore nuclei which may be arranged somewhat diagonally (Fig. 3) or in a linear row (Fig. 4). Subsequently, one of the megaspore nuclei retains the micropylar position while the other three shift to the chalazal end. They are separated by a large central vacuole. Besides the 1 + 3 arrangement (Figs. 6, 7), rarely there may be two nuclei at each pole. When this happens one nucleus of each pair is usually smaller than the other (Fig. 5).

Figure 8 represents a gametophyte where the three chalazal nuclei have partially fused. In Fig. 9, on the other hand, all the four megaspore nuclei are in division. This would lead to a 2 + 6 arrangement which is not uncommon and is characteristic of plants showing the Drusa type of development.

I did not observe fusion of the three chalazal spindles but presume that it does take place. A secondary 2-nucleate stage with the haploid micropylar and probably triploid chalazal nucleus is shown in Fig. 10. Sometimes the chalazal nucleus divides earlier than the micropylar resulting in a 3-nucleate condition (Fig. 11). After the division of the micropylar nucleus a secondary 4-nucleate gametophyte is formed (Figs. 12, 13). The chalazal nuclei frequently show several nucleoli. Their precocious division produces a 6-nucleate stage (Fig. 14) and the last nuclear division (Fig. 15) gives rise to the 8-nucleate embryo sac. The four micropylar nuclei organize into the egg apparatus and the upper polar nucleus (Fig. 17). The delimitation of the antipodal cells precedes the organization of the egg apparatus (Fig. 16). Thus, the development conforms to the Fritillaria type and the material from Denmark is not essentially different from that of Italy.

FERTILIZATION — The egg apparatus shows signs of degeneration soon after its organization and the synergids are the first to collapse (Figs. 18, 19). Gradually the place of the egg apparatus is occupied by the ingrowing nucellar cells. None of my preparations showed any trace of the pollen tube either in the tissue of the style or in the embryo sac, and there was no indication of syngamy or triple fusion.

ENDOSPERM — When the ' polar fusion nucleus ' divides, the egg apparatus and the antipodal cells have already degenerated. Free nuclear divisions continue until about 100 or more nuclei are produced. The endosperm nuclei take up a peripheral position and each shows several nucleoli. Occasionally, small enucleated vesicles develop from the peripheral cytoplasm and project into the central vacuole (Fig. 23). Wall formation in the endosperm is centripetal and is initiated when the

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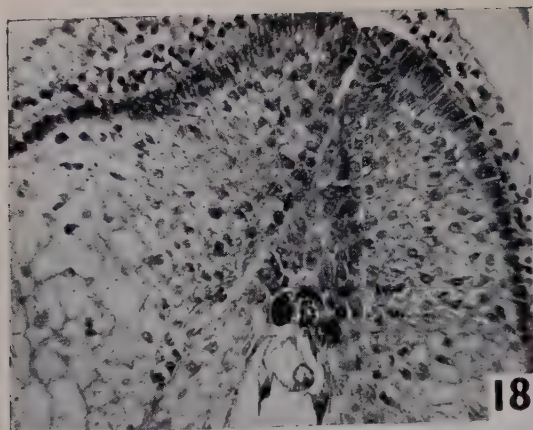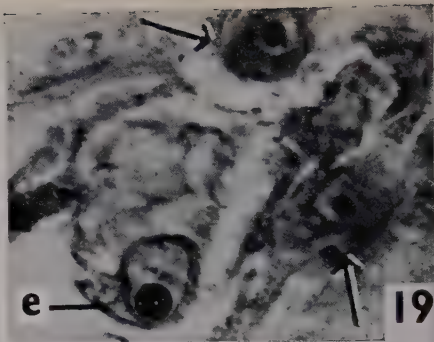
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nucellar embryos attain the early globular stage, and finally the entire endosperm becomes cellular (Figs. 25, 26). In the absence of triple fusion the development of endosperm must be assumed to be autonomous but due to lack of suitably fixed material chromosome counts could not be made to confirm this point.
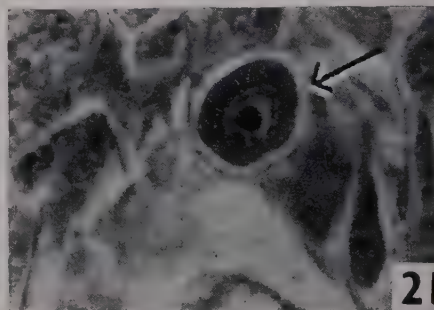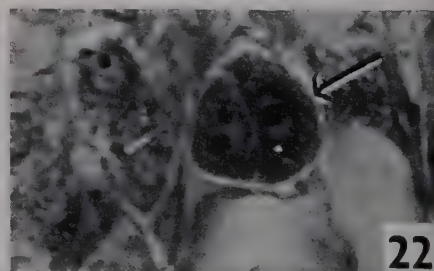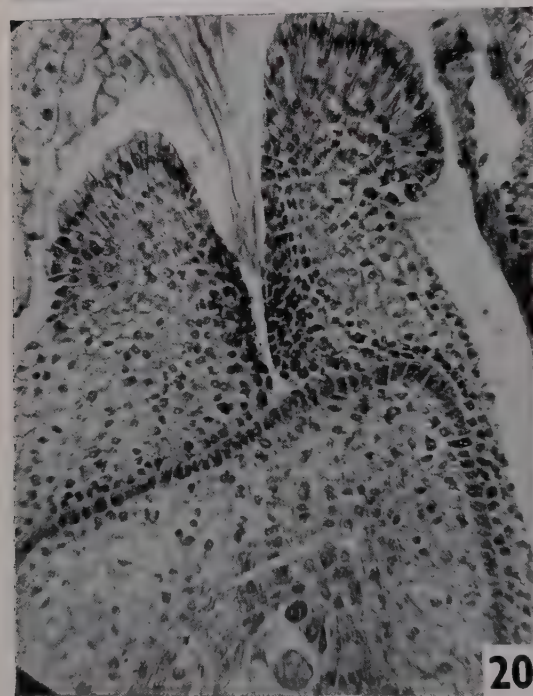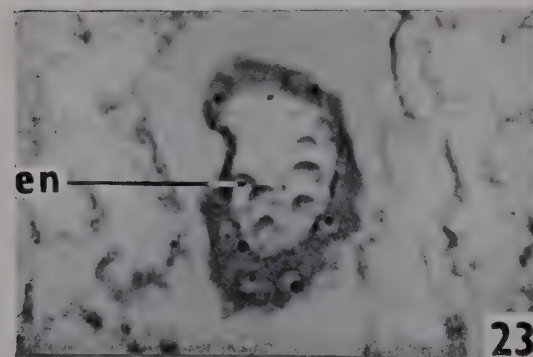
POLYEMBRYONY — In view of the degeneration of the egg, it seems likely that all the embryos are of nucellar origin.

A number of nucellar cells situated at the micropylar end become prominent. They are quite large and contain denser cytoplasm and prominent nuclei (Figs. 18-21). Sometimes they may be distinguishable as early as the primary 4-nucleate stage of the embryo sac. Such ' plasma-rich ' cells may also differentiate laterally and at the chalazal end (Figs. 27-30). Their contents round off (Figs. 21, 31), followed by a transverse division of the cell (Fig. 32). Sometimes the first division may be vertical or oblique (Figs. 22, 33, 34). The apical cell now undergoes a vertical division (Figs. 35, 36) resulting in a 3-celled adventive proembryo. With the division of the basal cell which may be transverse (Figs. 39, 40) or sometimes vertical (Fig. 37), the embryonal masses push their way into the cavity of the embryo sac (Fig. 24). Irregular divisions occur in the terminal cell (Figs. 37-40) resulting in a globular proembryo (Figs. 25, 26, 41). In a few cases, the derivatives of the basal cell simulated suspensor cells.

Even after the formation of globular proembryos additional nucellar cells may continue to differentiate and sometimes 2-13 embryos may develop concurrently. The shape and size of the embryos depend on spatial relationship and the same ovule may show a considerable range in their development.

The globular proembryo gradually develops into a heart-shaped and finally into a dicotyledonous embryo (Figs. 42-45).

SEED AND TESTA — At the primary 4-nucleate stage of the embryo sac each integument comprises three or four layers of cells (Figs. 46, 47). The outer integument is slightly swollen in the micropylar region and shows six to eight layers of cells. Later it becomes 8-12 layered in the micropylar region and its cells proliferate giving rise to the characteristic caruncle (Figs. 48, 50, 53, 54). The inner integument consists of 6-9 layers of enlarged cells (Fig. 49). Gradually the cells of its outer epidermis develop into sclereids (Fig. 52).

The dark brown seeds remain covered with remnants of the outer integument and display a prominent fan-shaped caruncle (Figs. 50, 51, 53, 54) which is much better developed in this than in other species of *Euphorbia*.

FRUIT AND PERICARP — At the secondary 2-nucleate stage of the embryo sac the ovary wall consists of 10-12 layers of parenchymatous cells (Figs. 56, 57). It shows numerous, long, unicellular hairs, each with a large spindle-shaped nucleus (Figs. 58-60). The differentiation into three zones — pc_1, pc_2, pc_3 — is similar to that in *Chrozophora* (Figs. 56, 62, 64; *see* also Kapil, 1956).

During the maturation of the pericarp, the outermost region (pc_3) becomes several layered and short protuberances appear on the surface (Fig. 62). These outgrowths assume the shape of large warts (Figs. 63, 64) which have numerous stomata. At the same time, most of the epidermal hairs fall off.

As compared to other species, the palisade-like cells (Fig. 65) of the middle region (pc_2) are also prominently developed and the inner epidermis (pc_1) consists of tangentially elongated cells.

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FIGS. 18-24 — Endosperm and Embryo (Photomicrographs; *e*, egg; *en*, endosperm nodules). Figs. 18, 20. Outline photographs for Figs. 19 and 21. × 200. Figs. 19, 21. Tips of embryo sacs enlarged to show 'plasma-rich' nucellar cells (indicated by arrows); the egg is also seen in Fig. 19. × 933. Fig. 22. Two-celled nucellar embryo. × 933. Fig. 23. Embryo sac showing enucleated endosperm nodules. × 300. Fig. 24. Nucellar embryos. × 400.

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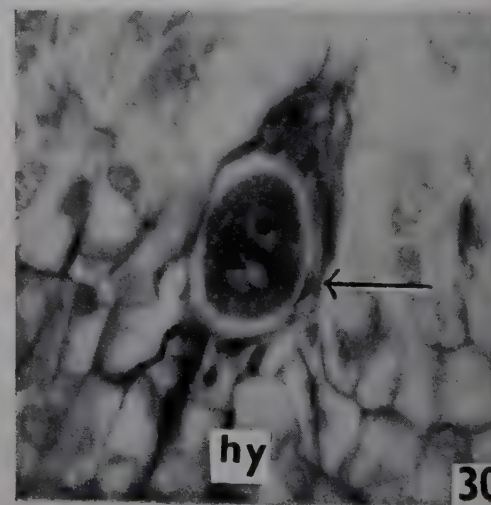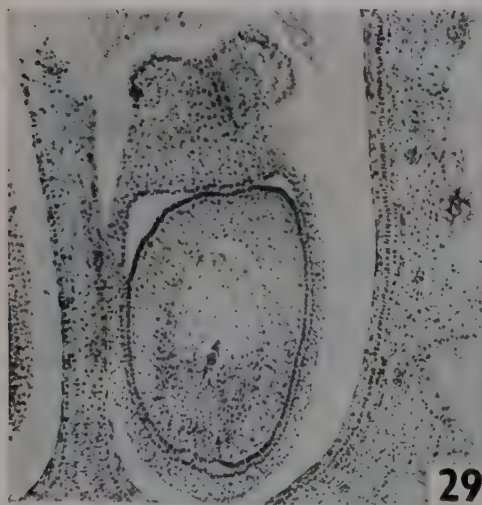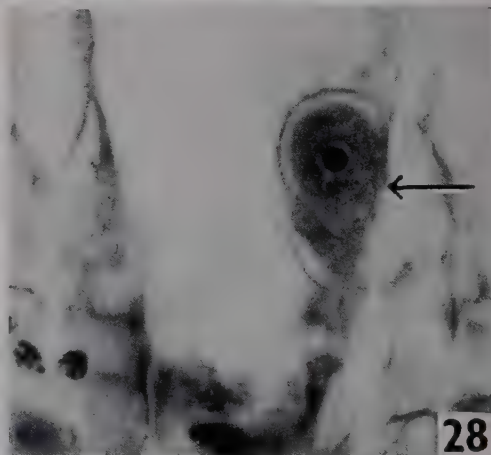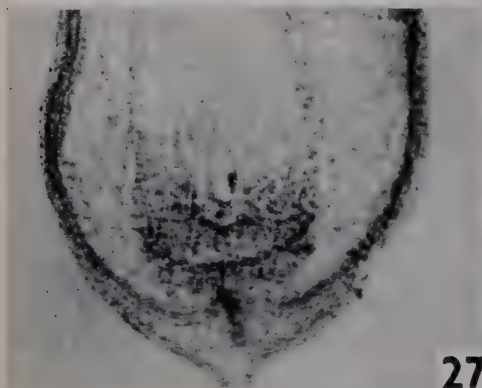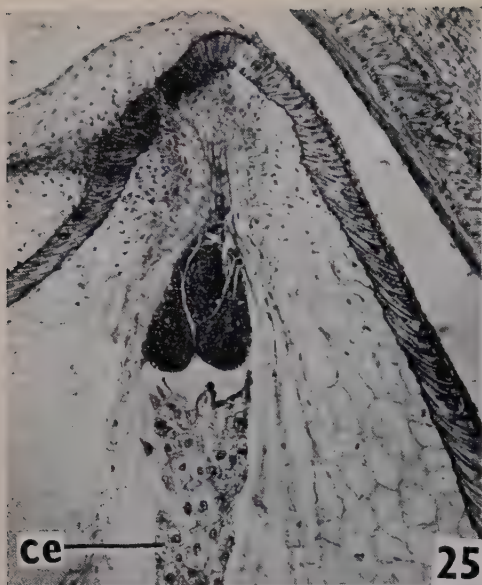
Discussion

Some of the important features concerning the female gametophyte, fertilization and polyembryony in *Euphorbia dulcis* may now be discussed in the light of the present reinvestigation.

FEMALE GAMETOPHYTE — Of the six species of *Euphorbia* showing a tetrasporic development of embryo sac (*see* Table 1) there are three which seem doubtful — *E. heterophylla, E. pulcherrima* and *E. virgata*. Sanchez (1938) reported the Adoxa type in *E. heterophylla* but on reinvestigation Maheshwari (1942) observed that the embryo sac is of the Polygonum type (*see* also Modilewski, 1910). Similarly, Dessiatoff (1911) recorded a 16-nucleate gametophyte in *E. virgata* while Modilewski (1911) reported Polygonum type (*see* also Schürhoff, 1926; Bohn, 1924). In *E. pulcherrima* also Donati (1912) described a 16-nucleate embryo sac but according to Sharma (1955) the development may be of the Adoxa or Polygonum type.

So far *E. dulcis* is the only species with the Fritillaria type of embryo sac. To quote Carano (1926): " I due grossi nuclei prodotti in seguito alla divisione eterotipica sono da principio separati da un fragmoplasto molto evidente, ciò che lascerebbe supporre la formazione di una membrana divisoria e la costituzione di una normale diade; ma poi il fragmoplasto si riássorbe completamente e i due nuclei rimangono nella medesima cavità cellulare." None of my preparations gave any indication of the formation of dyad cells, and in addition to the 1 + 3 arrangement of megaspore nuclei, a 2 + 2 arrangement was also observed. Carano (1926) considered the development in *E. dulcis* to be intermediate between the 8- and 16-nucleate types known in euphorbias.

FERTILIZATION — The absence of fertilization in *E. dulcis* is highly significant. Hegelmaier (1901, 1903) could not decide whether the embryo was formed as a result of fertilization, or parthenogenetic development of the egg. Carano (1926) stated that due to sterility of pollen the egg is never fertilized and my observations confirm this conclusion.

POLYEMBRYONY — According to Hegelmaier (1901, 1903), in *E. dulcis* 2 to 9 embryos may develop concurrently with the ' zygotic ' embryo. Only that arising from the egg appeared to be normal and possessed a 4-celled filamentous suspensor. The remaining embryos either developed from the synergids or the nucellar cells. Hegelmaier noted that most of the pollen grains were shrunken and contained only the microspore nucleus. He did not observe the pollen tube in the ovule and thought that fertilization was perhaps unnecessary, at least for the development of the adventive embryos. He was, however, unable to decide whether the unfertilized egg or the synergid gave rise to an embryo.

Carano (1925, 1926) pointed out that owing to the absence of fertilization the egg fails to develop. The synergids also

TABLE 1

NAME OF SPECIES	INVESTIGATOR	TYPE OF EMBRYO SAC
Euphorbia dulcis	Carano, 1926	Fritillaria
E. dulcis	Martinoli, 1940	Fritillaria
*E. heterophylla**	Sanchez, 1938	Adoxa
E. palustris	Modilewski, 1911	Penaea
E. procera	Modilewski, 1909	Penaea
E. procera	Schürhoff, 1924	Penaea
E. pulcherrima	Donati, 1912	'16-nucleate'
*E. pulcherrima**	Sharma, 1955	Adoxa
*E. virgata**	Dessiatoff, 1911	Penaea

*Doubtful cases.

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FIGS. 25-30 — Embryo (*ce*, cellular endosperm; *hy*, hypostase). Figs. 25, 26. Longisections of immature seeds; note three globular nucellar embryos in Fig. 25, and one globular and one heart-shaped embryo in Fig. 26, Fig. 25. × 100; Fig. 26. × 63. Figs. 27, 29. Outline photographs for Figs. 28 and 30. × 90. Figs. 28, 30. Enlargements of chalazal portions; note the 'plasma-rich' nucellar cells (indicated by arrows); in Fig. 30 the thick-walled cells of the hypostase are also distinguishable. × 933.
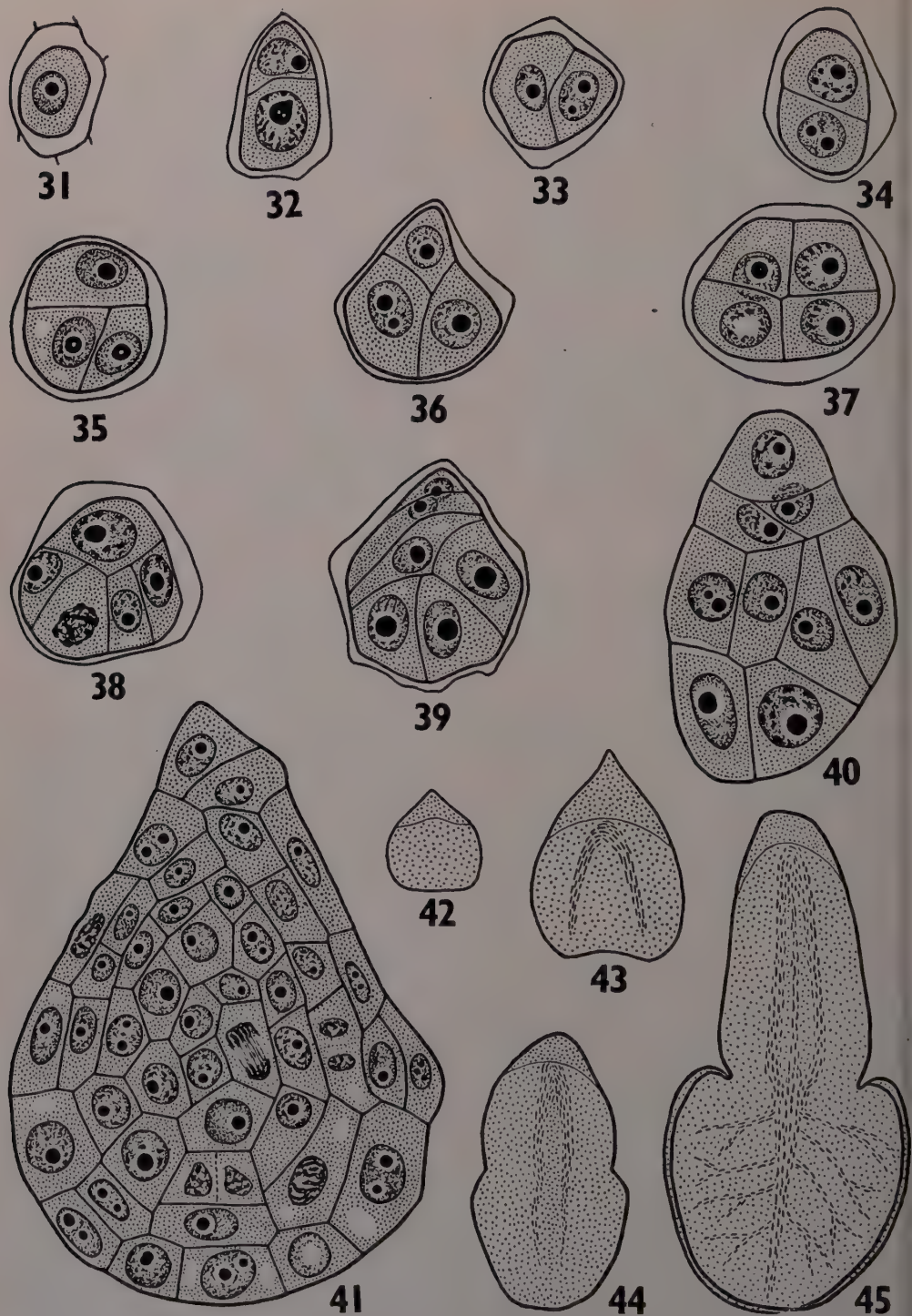
FIGS. 31-45 — Adventive embryos. Stages in the development of nucellar embryos (explanation in text); in Figs. 31-39 the original wall of the nucellar cell is also distinguishable. Figs. 31-41. × 913; Figs. 42, 43. × 120; Figs. 44, 45. × 60.
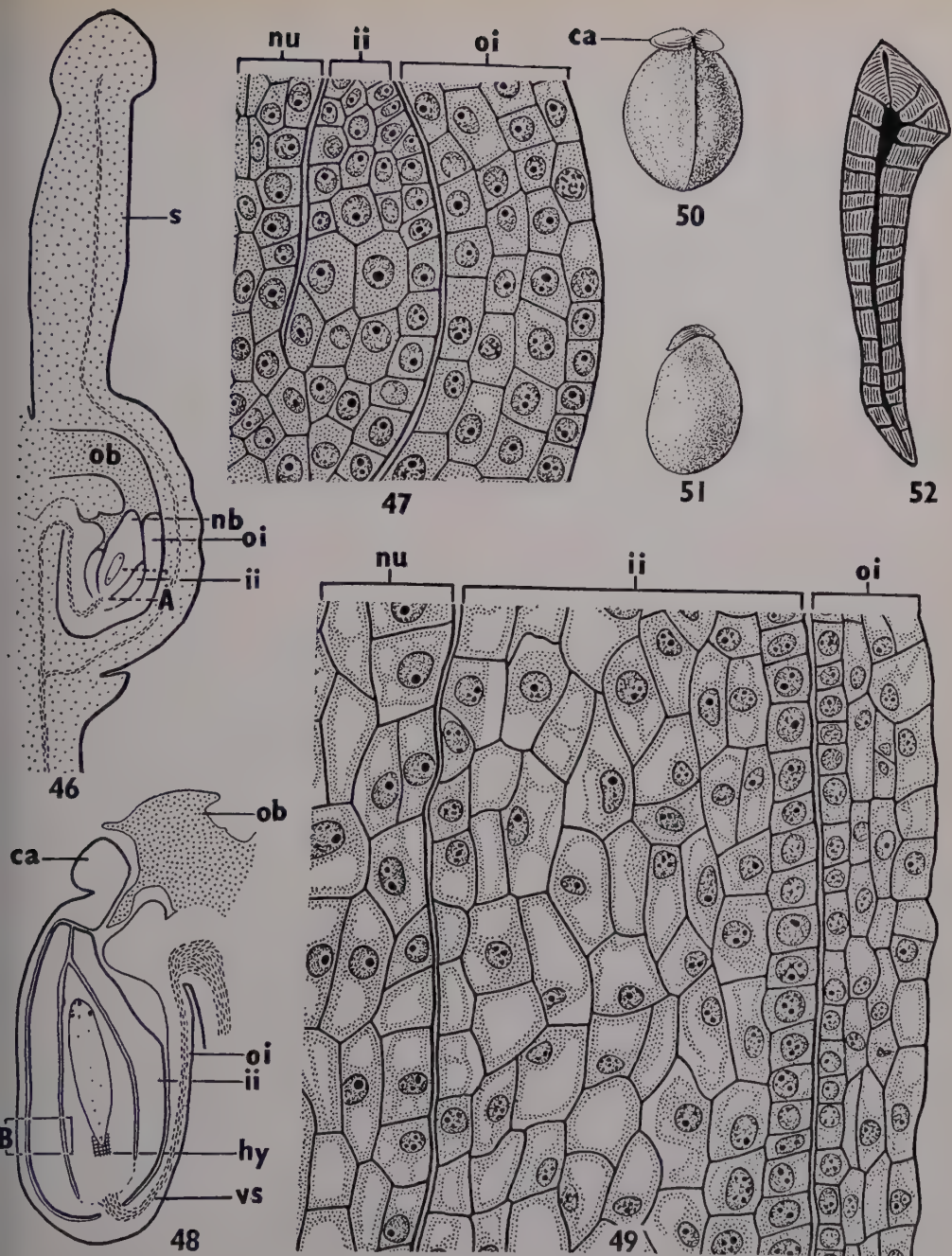
Figs. 46-52 — Seed and Testa (*ca*, caruncle; *hy*, hypostase; *ii*, inner integument; *nb*, nucellar beak; *nu*, nucellus; *ob*, obturator; *oi*, outer integument; *s*, style; *vs*, vascular supply). Fig. 46. Outline sketch for Fig. 47; the embryo sac is at the secondary 2-nucleate stage. × 60. Fig. 47. Enlarged view of integuments and nucellus from portion marked A in the preceding figure. × 586. Fig. 48. L.s. young seed (semi-diagrammatic). × 60. Fig. 49. Portion marked B in Fig. 48. × 586. Figs. 50, 51. Surface and side views of mature carunculate seeds. × 6. Fig. 52. Sclereid from outer epidermis of inner integument (macerated preparation). × 586.
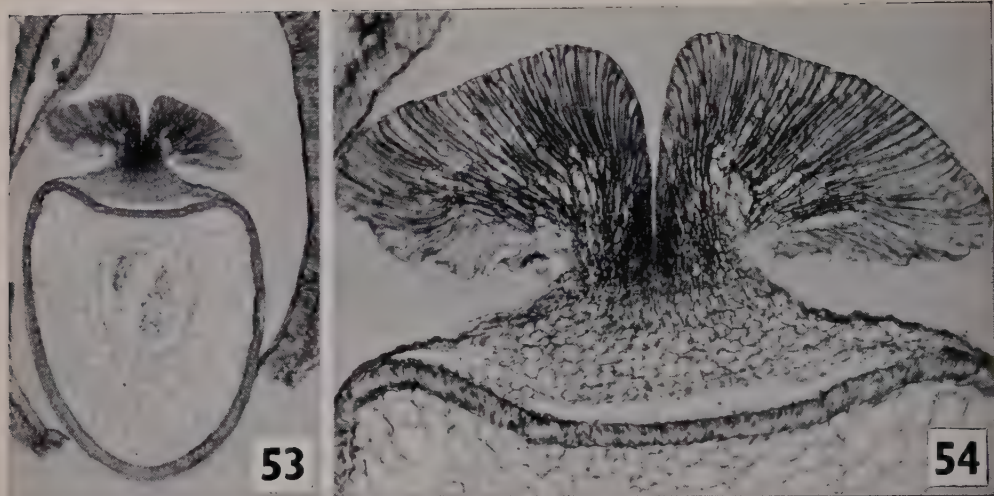
FIGS. 53-54 — Seed and Testa (contd.). Fig. 53. Longisection of nearly mature seed (photomicrograph). × 28. Fig. 54. Upper part enlarged to show the detailed structure of caruncle. × 91.

degenerate and do not give rise to an embryo. This I fully confirm. In fact, the egg apparatus degenerates quite early and Hegelmaier's report that the egg or the synergid may give rise to the embryo is erroneous.

According to Hegelmaier the nucellar embryos are always devoid of a suspensor. However, I have observed several nucellar embryos with suspensor-like cells. As a rule, the nucellar embryos do not show any regular sequence of development. The first division may be transverse, vertical or oblique, and not invariably transverse as interpreted by Johansen (1950). The second division may be at right angles or parallel to the first. Subsequent divisions are irregular.

The male flowers were rare in the material examined by me. Whenever present, the pollen grains were unhealthy. This tallies with the observation that the development of the nucellar embryos is independent of the stimulus of pollination and fertilization (see also Carano, 1926). It may be concluded that *E. dulcis* is an apomict.

The occurrence of polyembryony has been recorded in four more species of *Euphorbia*: *E. rosea*, *E. terracina*, *E. helioscopia* and *E. platyphylla*. The origin of the additional embryos is uncertain in *E. rosea* (see Robyns & Louis, 1942) and *E. terracina* (Ventura, 1940). Regarding *E. helioscopia* and *E. platyphylla*, Schürhoff (1926) states that one of the synergids produces the additional embryo (see also Robyns & Louis, 1942). This has, however, not been confirmed for *E. helioscopia* where the synergids degenerate at the time of or soon after fertilization (Kapil, 1958). *E. platyphylla* also deserves reinvestigation. Thus, as far as the genus *Euphorbia* is concerned the only established case of polyembryony is that of *E. dulcis*.

Summary

The inflorescence of *Euphorbia dulcis* exhibits considerable reduction in the number of male flowers. The anther shows four wall layers — epidermis, endothecium, middle layer and glandular tapetum. Tetrahedral, isobilateral and decussate tetrads are formed but no fertile pollen grains are produced.

The development of the female gametophyte conforms to the Fritillaria type. The egg apparatus and the antipodal cells are ephemeral. Syngamy and triple fusion are both absent. The endosperm
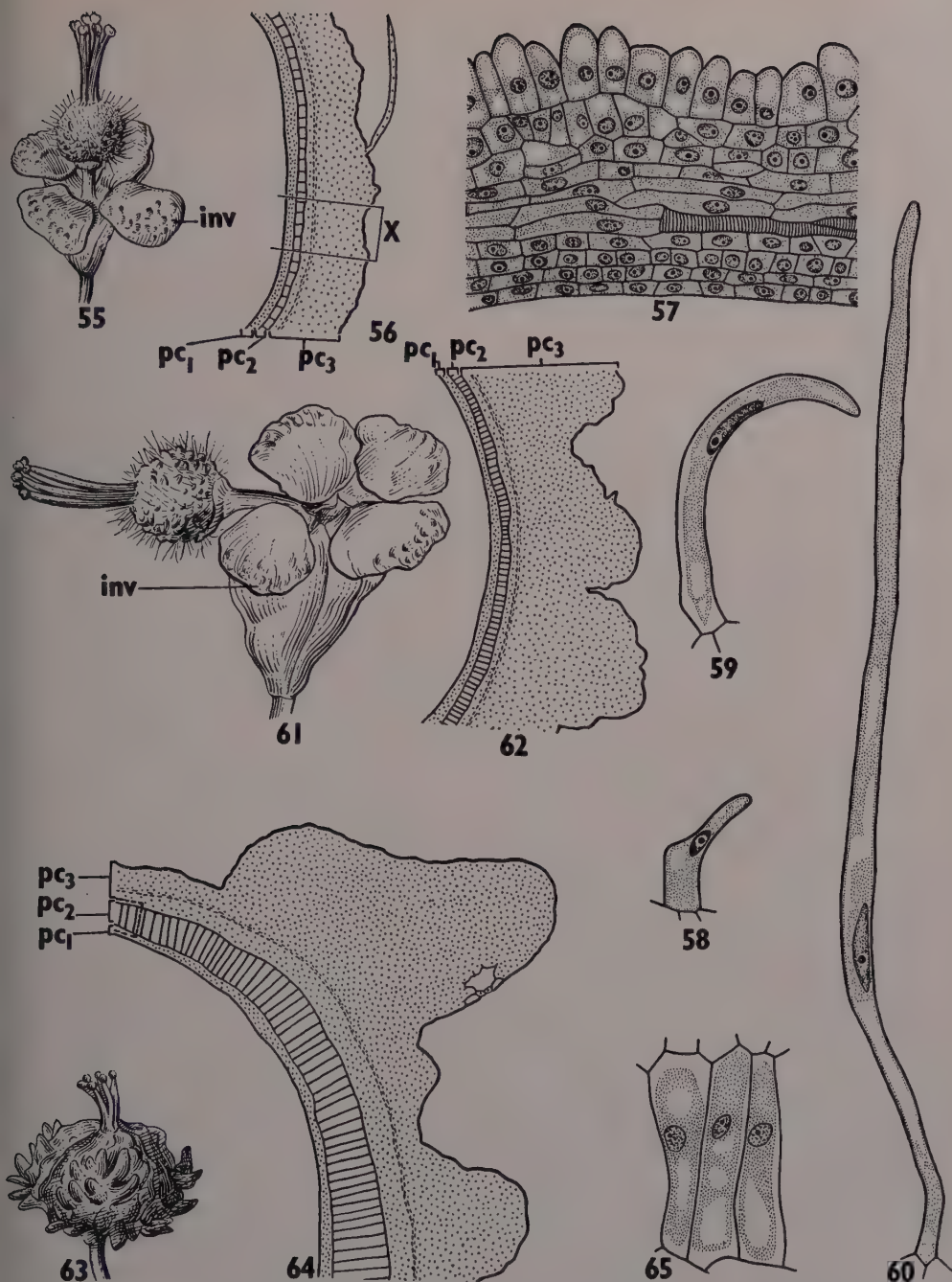
FIGS. 55-65 — Fruit and Pericarp (*inv*, involucre; pc_1, pc_2, pc_3, inner, middle and outer zones of pericarp). Fig. 55. Young ovary (the ovules are at the secondary 2-nucleate stage of embryo sac). × 5. Fig. 56. Portion of ovary wall almost at the same stage as in Fig. 55 (diagrammatic). × 125. Fig. 57. Enlargement of portion marked X in Fig. 56. × 440. Figs. 58-60. Hairs from the ovary wall. × 440. Fig. 61. Fertilized ovary. × 5. Fig. 62. L.s. pericarp showing differentiation of middle region (pc_2) into palisade-like cells (diagrammatic). × 125. Fig. 63. Mature fruit with lobed pericarp. × 5. Fig. 64. Delimitation of pericarp into three zones (diagrammatic). × 51. Fig. 65. Palisade-like cells from region pc_2 in Fig. 64. × 440.

is autonomous and Nuclear. Enucleated endosperm nodules have been observed. Wall formation in the endosperm begins at the early globular stage of the pro-embryo.

The zygotic embryo appears to be absent but a large number (up to 13) of nucellar embryos are produced from the micropylar or even chalazal or lateral regions of the nucellus and they push in into the cavity of the embryo sac. The sequence of development of the adventive embryos is irregular.

The formation of the seed and fruit proceeds normally as in other species of *Euphorbia*.

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SUR L'ONTOGENÈSE DE LA FLEUR MÂLE DE *WELWITSCHIA MIRABILIS* HOOKER

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Nous avons publié, en 1959, un mémoire étendu sur la structure et l'ontogenèse du cône et de la fleur femelles de *Welwitschia mirabilis* Hooker. Ce travail, intégré à une série d'études sur les Gnétales, poursuivies dans notre Institut depuis près de dix ans (Waterkeyn, 1954, 1959, 1960), imposait des recherches parallèles et comparatives sur le cône mâle et la fleur mâle (ou plus exactement "pseudo-hermaphrodite"). Préalablement à une publication intégrale, actuellement en préparation, nous présentons ici, avec une documentation photographique correspondante, quelques-uns des résultats déjà obtenus[1].

La Fig. 1 représente le sommet d'un cône en plein développement, disséqué de façon à laisser en place, par enlèvement des bractées axillantes, les 13 verticilles des plus jeunes ébauches florales.

La Fig. 2 montre, à un grossissement plus élevé, l'apex du même cône (*a*), les premières ébauches de bractées laissées en place (*b*), et les ébauches florales des verticilles fertiles impairs (3e, 5e et 7e), vues de haut. L'ébauche florale la plus jeune est cachée à l'aisselle de la troisième paire de bractées.

Les Figs. 3 et 4 représentent une même ébauche florale, celle du 8e verticille " fertile ", à partir du sommet, après dissection de cette ébauche et enlèvement des 4 pièces de son périanthe. La première (Fig. 3) est vue de haut, la seconde (Fig. 4) de côté et par sa face dorsale ou antérieure (c.à.d. en regard de la bractée axillante).

La Fig. 5 est une vue de haut de la fleur suivante (9e verticille), également dépouillée de son périanthe et assez grossie pour mettre en évidence la texture cellulaire.

La Fig. 6, enfin, représente une fleur plus développée, de nouveau en vue dorsale et privée de périanthe (11e verticille).

Aucune photographie de ces objets n'avait pu être obtenue et publiée jusqu'aujourd'hui.

Ces documents — et beaucoup d'autres — confrontés avec des séries de coupes microtomiques, réalisées suivant toutes les orientations utiles, démontrent notamment les faits suivants:

1° L'ébauche initiale est toujours semi-lenticulaire et biconvexe. Elle soulève successivement un verticille *latéral* de deux bractées étroites et libres, puis un verticille *médian* de deux bractées larges et connées, dont la postérieure recouvrira plus tard l'antérieure (Fig. 2, verticilles, 3, 5, 7). Cette origine du périanthe était déjà bien établie par nos devanciers (Hooker, 1863 ; Strasburger, 1872 ; McNab, 1873 ; Church, 1914, etc.).

2° Le centre de l'ébauche forme ensuite un " socle " ou " disque staminal ", partagé presqu'aussitôt en deux épaulements latéraux, qui représentent le troisième verticille de l'ébauche florale (Fig. 3, *s*). Chaque épaulement soulève ensuite, *simultanément* et sur ses bords, *quatre* protubérances (Fig. 4):

a) Deux de ces protubérances sont superposées dans le plan strictement latéral (" plan transversal " ou " tangentiel " de certains auteurs); elles occupent donc le milieu de chaque épaulement. Mais l'une d'elles est insérée un peu plus

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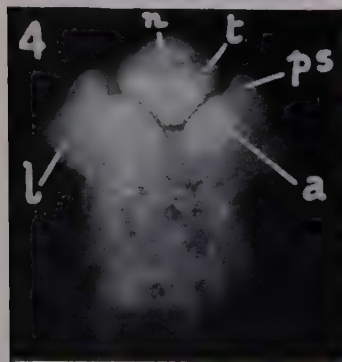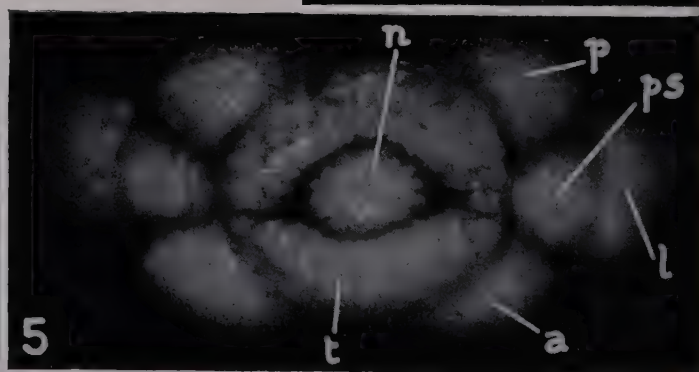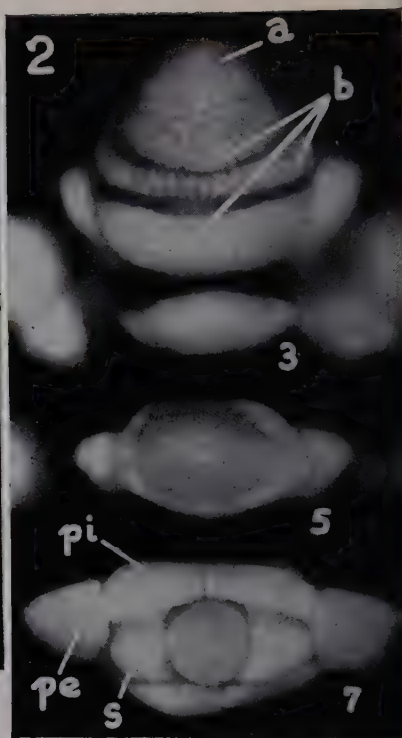
bas et orientée initialement vers l'extérieur et un peu vers le bas (*l*) ; elle deviendra une des deux étamines *latérales* de la fleur adulte (*l*, Fig. 6). L'autre, orientée vers le haut, s'effacera dans la suite et n'est que *pseudostaminale* (*ps*). Ces deux émergences ont notamment été confondues par McNab et Strasburger, mais reconnues, quoique peu clairement, par Church.

b) Les deux autres émergences de chaque épaulement occupent, sur celui-ci, une position respectivement postérieure et antérieure. Elles deviendront respectivement une des deux étamines *postérieures* et une des deux étamines *antérieures* de la fleur (*p*, *a*, Figs. 4, 6).

3° Sur une vue *polaire* de l'androcée, à ce stade (Fig. 3), les huit protubérances ne sont pas encore clairement distinctes ; en vue dorsale (Fig. 4), elles sont incontestables, sauf les émergences postérieures (ventrales), qui restent nécessairement cachées. Un peu plus tard et en utilisant un éclairage " rasant ", qui accentue le relief, les huit protubérances apparaissent très distinctement (Fig. 5, 9e verticille fertile du cône). Ce dernier document met bien en évidence la protubérance pseudostaminale (*ps*), l'étamine latérale (*l*) occupant un plan plus profond. Il démontre ensuite que les six étamines authentiques sont initialement bien distinctes à leur insertion sur le socle staminal sous-jacent. Il établit surtout la séparation initiale accentuée entre les deux étamines postérieures (*p*) et la séparation non moins accentuée entre les deux étamines antérieures (*a*). Aussi faut-il écarter l'opinion des auteurs qui voient, dans chacune de ces paires, le résultat de la *bifurcation* de deux étamines originelles,

l'une antérieure, l'autre postérieure, dédoublées secondairement dans le plan médian (Hagerup, 1934, Emberger, 1944, 1960).

4° L'ovule — destiné à l'avortement, comme on le sait depuis Hooker — délimite son unique tégument par un sillon strictement circulaire, continu et perpendiculaire à l'axe floral (*n*, *t*, Figs. 3, 4). Un peu plus tard et dès son accroissement vers le haut en " tube micropylaire ", ce manchon circulaire de tissu se partage en deux lobes, l'un dorsal, l'autre ventral (Fig. 5). Les lobes ne sont libres qu'au sommet du tube et formeront ultérieurement les deux lèvres du stigmate (*st*, Fig. 6).

Ces observations sont incompatibles avec l'idée d'*un* organe de nature foliaire, né dans le plan médian de la fleur, derrière le noyau ovulaire, et *enveloppant progressivement* ce noyau vers l'avant (Hagerup, 1934). Elles sont aussi en discordance — du moins sur le plan ontogénétique — avec l'interprétation générale du tégument ovulaire comme une *macrosporophylle*, interprétation défendue par le même auteur et par d'autres (Faegri, Emberger, etc.). Notre étude de la fleur ♀ nous avait déjà contraint à ce rejet, du moins pour le *Welwitschia* et le *Gnetum*.

5° La fleur nait exactement comme un strobile, et ces deux organes axillaires ne se différencient clairement qu'au cours de la croissance du verticille de l'androcée.

6° L'obliquité (à ± 45°) de l'axe floral sur l'axe du strobile, révélée par la Fig. 1, implique évidemment que des sections *transversales du strobile* ne peuvent donner que des sections franchement *obliques des fleurs*. Une ontogenèse de la fleur ♂,

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FIGS. 1-6 — *Welwitschia mirabilis*, cônes et fleurs ♂ (*a*, apex du strobile ; *b*, bractées axillantes du strobile ; 3-13 numérotation des fleurs, suivant le verticille " fertile " du cône, à partir de l'apex [les ébauches florales 1 et 2 ne sont pas visibles] ; *pe*, périanthe externe ; *pi*, périanthe interne ; *s*, socle staminal ; *l*, étamine latérale ; *a*, étamine antérieure ; *p*, étamine postérieure ; *ps*, protubérance " pseudostaminale " ; *t*, tégument ; *n*, nucelle ; *st*, stigmate.) Fig. 1. Sommet d'un cône en croissance, dépouillé de ses bractées axillantes ; 13 verticilles successifs d'ébauches florales. × 30. Fig. 2. Idem ; ébauches florales intactes des verticilles " fertiles " 3, 5 et 7. ×100. Figs. 3-6. Ébauches florales dépouillées de leur périanthe. Fig. 3. Fleur du 8e-verticille ; vue de haut. × 100. Fig. 4. Même fleur, face dorsale. × 100. Fig. 5. Fleur du 9e-verticille, vue de haut. × 200. Fig. 6. Fleur du 11e-verticille, face dorsale. × 70.

exclusivement basée sur des sections de ce genre (c'est le cas de Hagerup, 1934, et, partiellement, de Sykes, 1910, et de Lignier & Tison, 1912), ne peut en donner que des images trompeuses, génératrices d'interprétations inadéquates. Seules des sections *obliques de strobile* — et dont l'obliquité aura été soigneusement calculée — peuvent livrer, *à la fois*, de part et d'autre de l'axe du cône et dans le même ruban microtomique, des sections correcte-ment *transversales* de la fleur et des sections *longitudinales-axiales* de celle-ci, suivant le plan " latéral " (ou " transversal "). Quoique moins accusée que sur le cône ♂, cette obliquité avait déjà été vérifiée par nous (1959b) sur le cône ♀. Mais, pour plusieurs motifs, ses conséquences pratiques sont plus radicales ici. En fait, les sections transversales de strobiles ♂ se sont révélées pratiquement *inutilisables* pour une étude ontogénétique de la fleur.

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MICROSPOROGENESIS IN *EXOCHORDA RACEMOSA*

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Introduction

Exochorda racemosa, commonly known as pearl bush, is a deciduous ornamental shrub belonging to the family Rosaceae. Since its introduction from China many years ago, it has been widely scattered over the United States, being prized for its racemes of showy white flowers that appear in early spring. According to Darlington & Wylie (1956) the haploid chromosome number has been established as eight, but, beyond this, the literature does not reveal any further scientific investigation of the species.

Materials and Methods

Flower buds, collected from plants on the campus of Mississippi State University, were killed and fixed either in Navaschin's, Bouin's, or Carnoy's fluid, processed and embedded in the usual manner, and sectioned at 10-15 microns. Several stains were used, satisfactory results being obtained with both Heidenhain's haematoxylin and safranin-fast green. All photomicrographs were made with a thirty-five millimeter camera and enlarged for publication.

Development of the Microsporangium

The young anther contains four locules or microsporangia (Fig. 1). The hypodermal archesporium divides in the usual manner to form an outer primary parietal layer and an inner primary sporogenous layer.

By both periclinal and anticlinal divisions the primary parietal layer forms an endothecium, two middle layers, and a tapetum. Both middle layers, specially the inner one, are much smaller than either the endothecium or tapetum (Fig. 2).

Repeated division of the primary sporogenous tissue produces numerous, small, angular spore mother cells which are densely packed in the microsporangium (Fig. 3).

Prior to the beginning of meiosis in the microspore mother cells the tapetal cells begin to enlarge. Enlargement is accompanied by mitosis without cytokinesis, the result being that cells with two to eight nuclei are evident (Fig. 4). In most cases, the nuclei adhere closely so that there appears to be a fusion into either one large nucleus or into one large and one or more small (Fig. 5), a condition frequently noted in angiosperms, according to Maheshwari (1950). The tapetum is of the glandular or secretory type, the cells remaining in approximately their original position until degeneration is complete (Fig. 6). During breakdown of the tapetum, the large nuclear clusters are sometimes seen to separate into the original number of small nuclei. The two middle layers, specially the outer, persist after the tapetum has disappeared, fragments sometimes being apparent at the time of anther dehiscence.

The endothecium develops in the usual manner. By the time the tapetum has begun to disintegrate, the cells of the endothecium have grown to maximum size and fibrous thickenings have developed on the walls (Fig. 7). During the expansion of the endothecium, the epidermis becomes distorted except for the small cells near the area of dehiscence which become highly cutinized.

Meiosis

Prior to the initiation of prophase I the densely-packed microspore mother cells are compressed so that they are
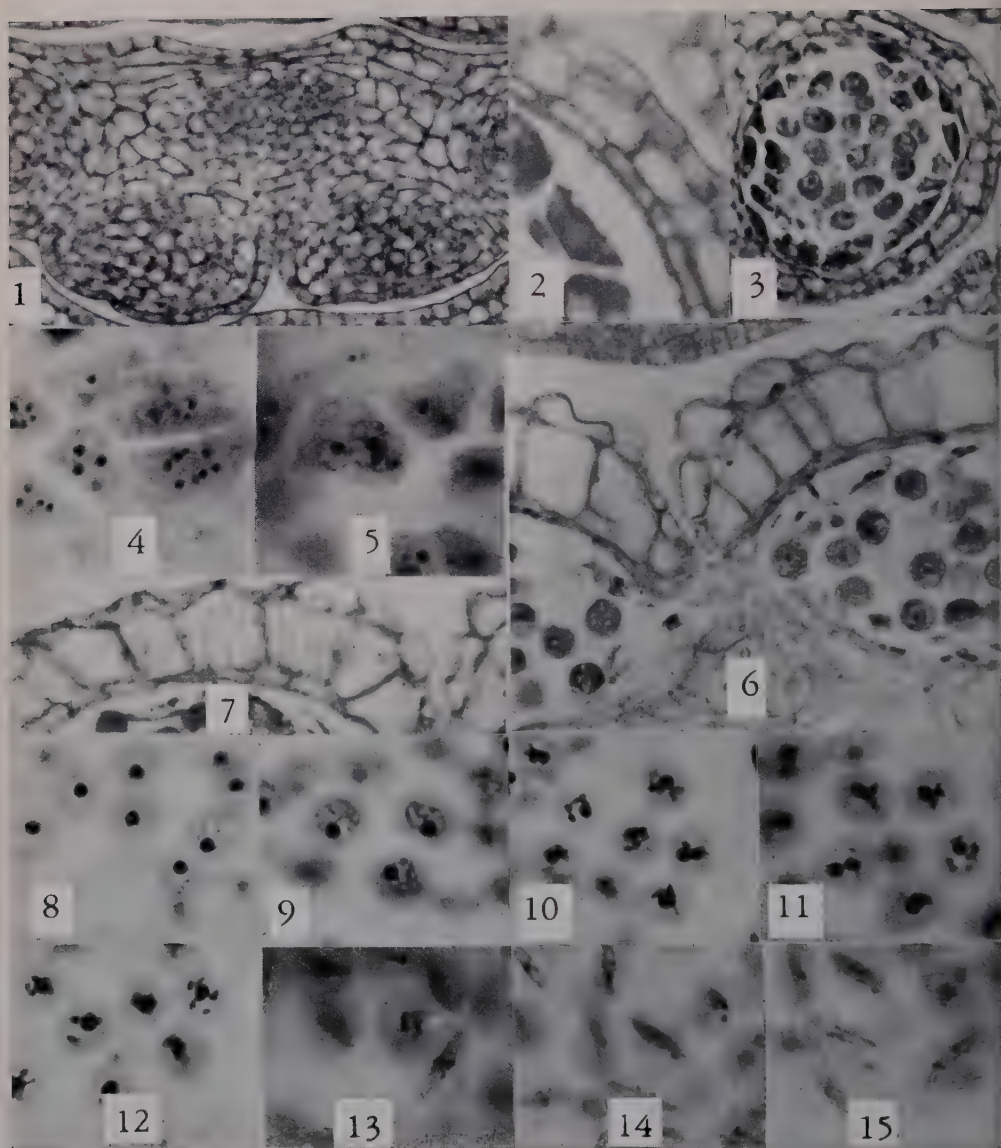
FIGS. 1-15 — Fig. 1. Cross-section of young anther. × 365.   Fig. 2. Tapetum pulled away, revealing two middle layers, endothecium, and epidermis.   × 625.   Fig. 3. Locule densely packed with microspore mother cells.   × 365.   Fig. 4. Poly-nucleate tapetal cells. × 625.   Fig. 5. Merging of tapetal nuclei into one large and one small nucleus. × 625.   Fig. 6. Degenerating tapetal cells; anther locules merging.   × 365.   Fig. 7. Fibrous thickenings on walls of endothecium.   × 365.   Fig. 8. Early prophase I. × 625.   Fig. 9. Pachynema.   × 625.   Fig. 10. Diplonema.   × 625.   Fig. 11. Early diakinesis.   × 625.   Fig. 12. Late diakinesis, bivalents moving towards nuclear membrane.   × 625.   Fig. 13. Metaphase I. × 625.   Fig. 14. Early anaphase I, " sticky " chromosomes tend to stretch.   × 625.   Fig. 14. Curving and elongating spindles during anaphase I.   × 625.

angular in outline. The cells are quite small, with correspondingly small nuclei and chromosomes. Early prophase I is normal in every respect, the homologues staining very lightly during leptonema and zygonema (Fig. 8). Synizesis, sometimes referred to as a fixation artifact, was not observed, even though three different fluids were used in fixation. At pachynema the thickened double threads, along with one prominent nucleolus, stand out in the nucleus of each cell (Fig. 9). Repulsion of the homologues at diplonema reveals the presence of numerous chiasmata (Fig. 10). As the bivalents shorten and thicken during early diakinesis they tend to become aggregated around the nucleolus (Fig. 11). Later, the bivalents move toward the nuclear membrane, free from the prominent nucleolus (Fig. 12).

At metaphase I the bivalents are arranged along the periphery of the spindle, rather than being scattered over the entire equatorial plate region. The spindle is long and narrow, the polar areas being greatly attenuated (Fig. 13). Because of the extremely small size of the chromosomes, kinetochore positions could not be accurately determined.

Metaphase I is of short duration, the homologues beginning separation soon after alignment on the equatorial plate. Because of either an inherent " stickiness " or the presence of numerous unterminalized chiasmata, the chromosomes present a stretched and distorted appearance at early anaphase I (Fig. 14). In some cases the clinging homologues appear to be aided in separation by an elongation of the spindle body, such an elongation often resulting in a curved spindle (Fig. 15). Once separation is complete, the dyads move to the poles without interruption. During anaphasic movement the chromosomes maintain their original peripheral positions, the tapered ends of the spindle widening ahead of the advancing chromosomes which reach the poles arranged in a circular pattern having a diameter essentially the same as that of the metaphase plate (Fig. 16).

For a short time after reaching the poles the chromosomes maintain their circular pattern, appearing in lateral view as dark streaks during early telophase I (Fig. 17). With the reappearance of nucleoli and nuclear membranes, the chromosomes slowly revert toward an interphase condition, fairly distinct chromosome bodies still being apparent at mid-telophase I (Fig. 18). By late telophase I the nucleoli are quite prominent and the chromosomes have lost much of their characteristic appearance (Fig. 19).

Prophase II occurs in the usual manner, the disappearance of nucleoli and membranes being accompanied by the appearance of distinct chromosomes. At metaphase II the spindles may lie in the same plane (Fig. 20) or in different planes (Fig. 21). As in prophase I, the spindle is long and narrow with pointed polar tips. Anaphasic separation occurs without disruption, the daughter chromosomes, as in anaphase I, moving along the periphery of the spindle. Telophase II follows the customary pattern, the chromosomes aggregate at the poles (Fig. 22), nucleoli and nuclear membranes reappear, and there is a return to the interphase condition.

Cytokinesis

Cytokinesis is of the simultaneous type, occurring by centripetal furrowing. Though a prominent spindle occurs during metaphase I and persists through telophase I, there is no evidence of cell plate formation. During very early telophase I the spindle material appears to expand in the equatorial region, continuing to the point of bulging the membrane of the spore mother cell (Fig. 23). Immediately thereafter, slight indentations occur in the areas of bulging (Fig. 24), giving the appearance that cytokinesis has begun. However, such furrowing is short-lived, the spindle and constrictions disappearing as the telophase nuclei prepare for division II. A similar condition was reported by Farr (1918) in *Magnolia*, the depth of constriction being greater than that observed in *Exochorda*.

Prominent spindles without cell plate formation occur in metaphase, anaphase, and telophase II. During reorganization of the telophase nuclei simultaneous centripetal furrowing, accompanied by vacuole formation, occurs (Fig. 25), each
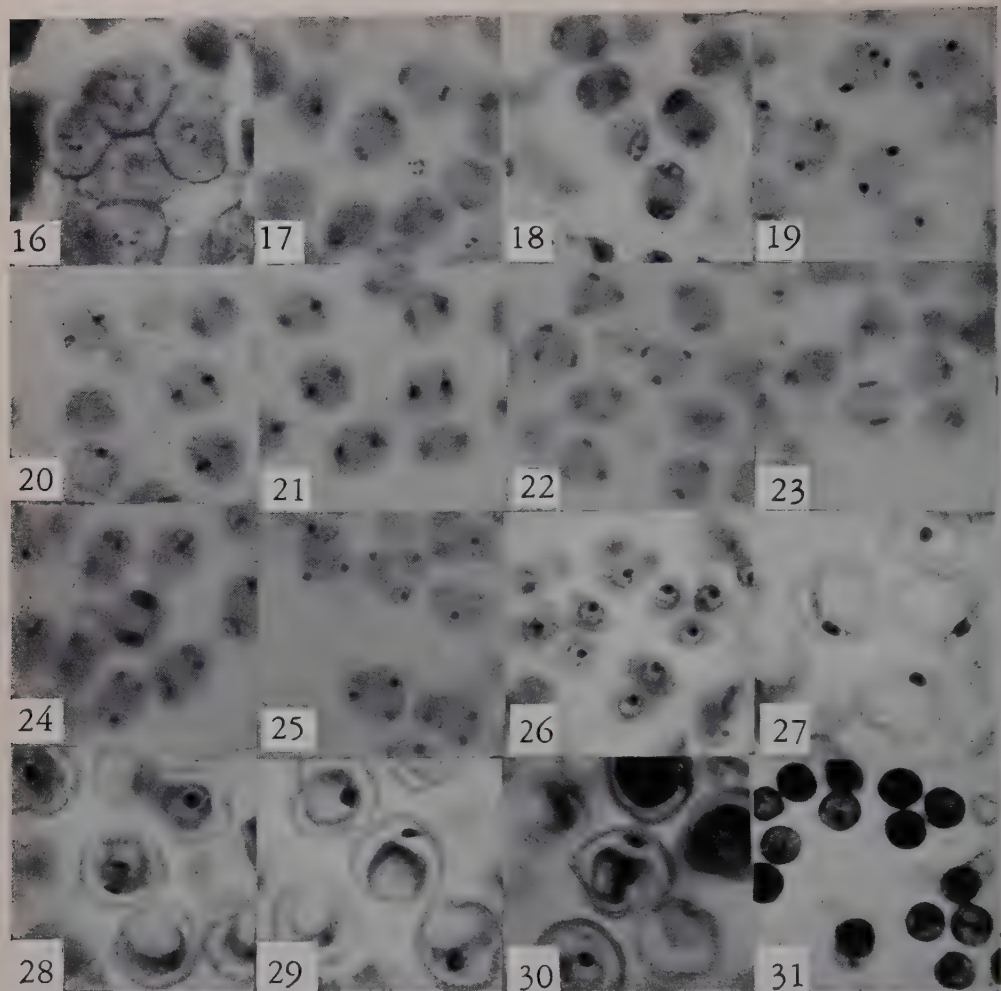
Figs. 16-31 — Fig. 16. Late anaphase I, polar view, seven of the eight dyads arranged in circular pattern on periphery of spindle. × 625. Fig. 17. Early telophase I. × 625. Fig. 18. Mid-telophase I. × 625. Fig. 19. Late telophase I, nuclei reverting to interphase state. × 625. Fig. 20. Metaphase II, spindles in same plane. × 625. Fig. 21. Metaphase II, spindles in different planes. × 625. Fig. 22. Very early telophase II. × 625. Fig. 23. Outward bulging of spindle area during early telophase I. × 625. Fig. 24. Temporary constriction furrow appearing during telophase I. × 625. Fig. 25. Simultaneous cytokinesis by furrowing, accompanied by vacuole formation, in late telophase II. × 625. Fig. 26. Tetrahedral arrangement of microspores. × 625. Fig. 27. Central vacuole pushes early prophase nucleus near microspore wall. × 625. Fig. 28. Early telophase in microspore nucleus, no cell plate evident. × 625. Fig. 29. Cytokinesis by furrowing. × 625. Fig. 30. Small, spindle-shaped generative cell cut off near wall. ×625. Fig. 31. Pollen grains at time of anther dehiscence. × 625.

nucleus with its surrounding cytoplasm being cut out as a three-angled microspore. Castetter (1925) observed a similar combination of furrowing and vacuole formation during cytokinesis in *Melilotus*

alba. Even though the metaphase II spindles often lie in the same plane, there is no evidence of an isobilateral arrangement of microspores. By the time cytokinesis occurs, the nuclei have shifted so

that the microspores commonly show a tetrahedral arrangement (Fig. 26).

Microspore Development

The young microspores, initially three-angled, gradually become rounded as they enlarge preparatory to nuclear division. During prophase a large vacuole arises in the centre of the microspore, pushing the nucleus and surrounding cytoplasm to a position near the microspore wall (Fig. 27). Division of the microspore nucleus occurs in the usual manner. The spindle is relatively symmetrical (Fig. 28), no blunt wallward pole being observed as reported by Geitler (1935). No cell plate appears during telophase, the spindle material disappearing as furrowing cuts off a small spindle-shaped generative cell near the microspore wall (Figs. 29, 30). Thus, at the time of dehiscence of the anthers, each pollen grain contains a small generative cell and a large tube cell and has three germ pores, one pore usually being more prominent than the other two (Fig. 31).

Summary

The young anther has four locules, each densely packed with small microspore mother cells. The anther wall consists of an epidermis, an endothecium, two middle layers, and a tapetum. Mitosis without cytokinesis produces polynucleate tapetal cells, the nuclei sometimes merging. The tapetum remains in approximately its original position until degeneration is complete. Prophase I and metaphase I occur in the usual manner. A " stickiness " of chromosomes is evident at anaphase I, the spindle apparently elongating and/or bending as an aid to homologue separation. A quickly-arrested " false " cytokinesis occurs at telophase I, real cytokinesis occurring by simultaneous furrowing, accompanied by vacuole formation, at telophase II. The microspores are tetrahedrally arranged. At the time of anther dehiscence each pollen grain contains a generative cell and a tube cell and exhibits three germ pores.

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MORPHOGENETIC STUDIES ON THE FERN *TODEA BARBARA* (L.) MOORE — I. LIFE HISTORY

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Introduction

Todea (Willd.) has long been regarded as a distinct genus of the Osmundaceae. This family, considered to be the most primitive of the Filicales, occupies a position transitionally between eusporangiate and leptosporangiate ferns (Bower, 1926).

The family is usually divided into three genera; *Osmunda*, *Todea*, and *Leptopteris*. However, some disagreement exists as to the status of *Leptopteris*, whether it should be classified as a separate genus or be grouped as a subgenus of *Todea*.

Recent investigators have favoured the division of the Osmundaceae into three genera; *Osmunda*, *Todea*, and *Leptopteris* (Diels, 1936; Christensen, 1938; Copeland, 1947; Lawrence, 1951).

Todea (Willd.), unlike the genus *Osmunda* which is cosmopolitan, is disjunctive in its distribution. The genus is monotypic and composed of a single species, *Todea barbara* (L.) Moore, which is found in Australia, New Zealand, and South Africa (Lawrence, 1951). This species was used exclusively throughout the present study.

In the course of studies conducted in these laboratories to determine the relationship of the immediate environment to the growth and differentiation of fern embryos, certain features of the fern *Todea barbara* became evident which suggested the possibility of its use as experimental material for this type of study.

1. Gametophytes can be readily grown from spores and maintained in sterile culture on a synthetic medium for long periods — at least ten years so far in the Harvard Laboratories.

2. Fertilization in this fern, in contrast to others, is highly successful. Each prothallus studied bears at least one and, in the majority of cases, several functional embryos at a time and may produce successive embryos over a long time, even years.

Before embryological studies could be initiated, it was necessary to examine critically the various stages in the life history of this fern, which led to the formation of the embryo and its subsequent growth.

A large gap in the life history of *Todea* is seen to exist in the absence of a detailed study of the embryology of this fern and the stages of fertilization leading to the development of the embryo. Although previous studies have been conducted on certain stages in the life cycle of the plant (Luerssen, 1874; Stokey & Atkinson, 1956), these have been largely carried out utilizing prothalli grown on crockery, peat, or soil. None of these stages has been studied on prothalli grown in sterile culture.

It is, therefore, the purpose of this study to follow various stages in the life history of *Todea barbara*, as they occur in culture, beginning with the development of the prothallus and the inception of sex organs through the growth of the fertilized embryo. This study is intended to serve as a background and a basis of comparison for further morphogenetic studies.

Materials and Methods

ORIGIN OF GAMETOPHYTES—This investigation was carried out utilizing exclusively gametophytes which had been grown in sterile culture from spores following essentially the methods described by

Nebel (1946) and Hurel-Py (1950), etc. A plentiful supply of *Todea barbara* gametophytes was available for this study and by repeated sub-culturing of the original stock, a uniform clone of material was continually produced. The initial cultures had been previously established by germinating sterilized spores on a synthetic medium following the procedure of Steeves *et al.* (1955).

The medium employed for the germination of spores consists of Knudson's solution of mineral salts (Knudson, 1925), modified by using half the original quantity of salts according to the formula of Steeves *et al.* (1955) as follows:

Water (glass redistilled)	1000 cc
Ca(NO$_3$). 4H$_2$O	500 mg
(NH$_4$)$_2$SO$_4$	250 mg
MgSO$_4$.7H$_2$O	125 mg
K$_2$HPO$_4$	125 mg

In addition, 10 mg of ferric citrate and 1 ml of Nitsch's trace element solution (Nitsch, 1951), supplemented by the addition of 25 mg cobalt chloride, was added per liter of solution. To this solution, 0·25 per cent sucrose was added and the pH adjusted to 5·5. The medium was solidified by the addition of 0·8 per cent washed, shredded agar and poured into pyrex culture tubes of 18 mm diameter. The tubes were plugged with non-absorbent cotton and sterilized by autoclaving for twenty minutes at fifteen pounds pressure. The sterile tubes were then placed in tilted racks and allowed to harden to produce a slanted surface on which the spores could be planted.

The planting of spores and all subsequent culturing was carried out in a transfer room previously sterilized by ultraviolet light. All cultures were maintained in a room at a temperature of 25°C $\pm$ 1° and kept under twelve hours of light supplied by a combination of fluorescent and tungsten lamps.

PREPARATION FOR EMBRYO STUDIES — For the embryological investigation, sterile gametophytes were removed from the nutrient in the tubes in a previously sterilized transfer room. The gametophytes were carefully examined individually under a dissecting microscope at a magnification of 90X for any indication of earlier fertilization, often caused by water which has condensed on the inside of the tubes. Those gametophytes which at this time bore fertilized eggs were eliminated from this study. Gametophytes with mature antheridia and archegonia were placed in a sterile Petri dish and covered with sterile water. To insure adequate time for the fertilization process to take place, the gametophytes were kept covered with water for three hours. After this period, the gametophytes bearing fertilized eggs were transplanted to Petri dishes containing a modified Knudson's medium (Steeves *et al.*, 1955) with 1 per cent sucrose and allowed to develop under controlled light and temperature conditions.

Gametophytes were collected and examined at daily intervals over a period of one to thirty-five days after fertilization.

METHODS OF EXAMINATION — In the course of routine examination of the developing embryo, it was found possible to remove successfully the fertilized egg from the enveloping gametophytic tissue without injury. This could be accomplished from the fourth day after fertilization up to the twentieth day. The operation was carried out under a dissecting microscope utilizing both top and bottom lighting, at magnifications of 90X-160X. Gametophytes to be examined were placed bottom up in a drop of liquid nutrient medium in depression slides which had been coated with a thin film of paraffin. Insect pins were then inserted into each end of the prothallus and pressed into the paraffin, thereby keeping the prothallus stationary. Extremely fine, yet sturdy knives, needles, and other surgical instruments were prepared from very fine insect pins by careful sharpening on an oil stone under the dissecting microscope. These instruments proved invaluable in carrying out the delicate operation to be described.

By cutting the protruding neck of the archegonium from the prothallus, the developing embryo could be seen in its early stages surrounded by a two-layered calyptra. One pin, preferably curved, was inserted into the calyptra at the point where the archegonial neck had been previously removed. With a certain amount of practice, this could be accomplished without damage to the embryo.

Once the pin was inserted, a slow even pull succeeded in separating the embryo on one side, from the calyptra and surrounding tissue. In many of the operations carried out, it was at this stage that the embryo, unimpeded by its restraining surroundings, floated free from its position in the venter. In other cases, a slight pressure on the prothallial cells succeeded in freeing the embryo. Only in the later stages, twenty or more days after fertilization, was it ever necessary to force the embryo from its position. In these cases, a slight pressure with the blunt side of a microneedle against the embryo itself was enough to free it. Embryos isolated in this way were transferred to slides by means of a micropipette and examined without cover-glasses under a microscope at magnifications of 600X to 1250X. By leaving a portion of the calyptra adhering to the embryo, a point of reference for orienting the embryo was established.

This method of examination proved to be unequalled, particularly in determining the appearance of the embryo in its early stages and the planes of the first divisions.

For detailed microscopic examination of the various stages leading to the development and growth of the embryo, selected prothalli were fixed in Craf 3 fixative (Sass, 1951) for twenty-four hours, washed overnight in running tap water, and dehydrated in an ethyl alcohol-n-butyl alcohol series (Pratt & Wetmore, 1951). The tissue was then embedded in a rubberized paraffin, and sectioned at ten micra on a rotary microtome. The sections were mounted on slides and stained with Haidenhain's iron-alum haematoxylin, using a 1 per cent solution of safranin in 50 per cent ethyl alcohol as a counter-strain.

All photomicrographs were taken with a 35 mm single lens reflex camera (Exacta), using suitable extension tubes and microscope attachment. Kodak Panatomic X film was employed and developed in Kodak D-76 solution, according to the recommended procedure.

Observations and Results

MORPHOLOGY OF THE SPOROPHYTE — The sporophytic plant of *Todea barbara* is distinguished by possessing a large, slow-growing, heavy, upright stem which in the wild state is reported sometimes to extend a meter or so in length. Bower (1926) called attention to the fact that the axis is covered by persistent leaf bases arranged in a dense spiral, and is anchored to the soil by numerous dark-coloured roots which originate in close relation to the bases of the crowded leaves. Together they form a covering which hides the upright stem.

The fronds in this species are mostly bipinnate, though tripinnate leaves are known on older plants. The most striking feature of *Todea* and one which separates it from other Osmundaceous ferns is the noticeable absence of fronds which are entirely or partly given over to sporangial production, as are found in species of the genus *Osmunda*. In *Todea barbara*, there is no marked difference between sterile fronds and sporophylls. The sporangia are borne on the under surface of these non-specialized leaves, especially on the lower pinnae. The sporangia on *Todea* are of the Osmundaceous type, large, numerous, thick-stalked, and are arranged in compact clusters along the veins of the lowermost pinnae. In *Todea*, therefore, the sporangia are typically superficial, differing in this respect from the terminal position of the sporangia in *Osmunda*.

At maturity, the sporangia are pear-shaped. The walls of the sporangia are composed of thin-walled cells except for a group of polygonal, thick-walled cells in a lateral position near the distal end which serve as an annular patch. Dehiscence is by a vertical slit originating at the patch and extending over the top of the sporangia and down the other side. At the time of dehiscence, the spores are liberated, the number from a single sporangium having been estimated to be around 512 (Bower, 1926).

GROWTH OF THE PROTHALLUS — The germination of the spores of various species of *Osmunda* and *Todea* has been previously investigated by a number of workers (Stokey & Atkinson, 1956; Luerssen, 1874; Orth, 1936; Kny, 1872; Campbell, 1892). From their results, it appears that the stages of spore germination for the various members of the

family is similar. Germination of the spores in water or on damp soil takes place usually in one to two days (Stokey & Atkinson, 1956; Campbell, 1892), although Luerssen (1874) records six to seven days for the germination of *Todea* spores and Orth (1936) four days for germination of *Osmunda* spores on a nutrient agar. The protruding germ tube of the young prothallus undergoes a slight period of elongation before the first division occurs which separates a large prothallial cell from the smaller first rhizoid. This primary stage is closely followed by the occurrence of a number of divisions in the prothallial cell which leads to the formation of a plate of tissue. The filamentous stage of prothallial growth which is characteristic of many ferns growing in nature (Williams, 1938) and in culture (Hurel-Py, 1950) is not a conspicuous event in the growth of the prothalli of *Todea barbara*. Although the filamentous stage can be produced by crowding in growing prothalli of various species of *Osmunda* and *Todea*, or may even be permanent in certain ferns, as *Aneimia*, this is not usually a significant nor a prolonged event in the life history as it occurs either in nature or in sterile culture.

Soon after the filamentous stage, there follows the formation of a plate of cells, with the early setting off of an apical cell which begins cutting off cells on either side causing an increase in length of the prothallus. The continued activity of the apical cell results in the production of marginal lobes causing the thallus to assume the characteristic reniform or heart shape.

The apex is now situated between terminal lobes of the prothallus and can be distinguished by the character of its meristematic cells. As growth of the prothallus proceeds, the cells behind the apex divide parallel to the surface to form a band of more than one cell in thickness, commonly referred to as the " midrib " or " cushion ". Numerous developing rhizoids on the under side of this median cushion present the first visible evidence of differentiation in this relatively simple plant.

Initiation of sex organs then proceeds, although in the cultured gametophytes of

Todea it is common to find young, heart-shaped prothalli which have not yet developed an extensive midrib bearing numerous functional antheridia produced along the margins and on the central regions of the thallus. Small prothalli are characteristically formed as outgrowths from the parent thallus. Many instances have previously been reported of a marked tendency on the part of the prothalli of the Osmundaceae and other ferns to multiply by the production of secondary prothalli arising as superficial outgrowths from the primary prothallus (Kny, 1872; Campbell, 1892; Stokey & Atkinson, 1956). These may become separated and so give new prothalli, or they may grow, developing sex organs while attached to the original prothallus and so produce a variously branched, compound structure. These older prothalli and newly regenerated outgrowths can be conveniently divided and subcultured repeatedly giving rise to new colonies of prothalli, as described for other species by Hurel-Py (1950).

INITIATION AND DEVELOPMENT OF SEX ORGANS — The antheridia are the first organs to develop on gametophytes of *Todea barbara*. They are generally formed on the basal portion of the cordate prothallus arising from superficial cells along the margins, although it is not at all uncommon to find numerous antheridia also developing both anteriorly and posteriorly on the lobes of the thallus.

The beginning of antheridial development is generally noted by the marked protrusion of a marginal cell of the cultured prothallus (Fig. 1). The primary division of the bulging cell is commonly periclinal (Fig. 1), less often, oblique. This first division separates the hemispherical antheridial initial from the basal cell.

The primary division in *Todea*, as in many of the other leptosporangiate ferns, does not separate the fertile spermatogenous tissue from the sterile covering tissue (Foster & Gifford, 1959). Rather, it is not until several sterile cells have been derived from the antheridial initial that the primary spermatogenous cell is formed.

The antheridial initial formed, as a result of the first division, subsequently divides by an oblique anticlinal division

which intersects the first formed wall (Fig. 2). The formation of this wall is closely followed by another anticlinal division on the same side and parallel with the first, or by an oblique division which has its origin on the other side and succeeds in cutting out a wedge-shaped cell (Fig. 3). The developing antheridium at this stage shows two oblique antheridial walls intersecting the wall formed by the primary division. In this material a number of anticlinal divisions had occurred before another periclinal division took place. The second periclinal division separated a primary spermatogenous cell from the surrounding sterile tissue (Fig. 4).

The outer sterile cells then proceed, by a series of curved anticlinal and periclinal divisions, to form the indefinite, but more than one-cell thick, wall patterns characteristic of the mature antheridia of the Osmundaceae. The primary spermatogenous cell usually divides first by a vertical wall, although in some of the material examined, the first division was slightly inclined from the horizontal. A second wall, formed in the same plane as the first but at right angles to it, divides the future sperm cells into four. The quartet of spermatogenous cells is usually soon transformed into eight by the formation of a horizontal wall, although the plane of this division varies somewhat. Further stages in some of the antheridia observed appeared to follow closely a regular segmentation pattern by anticlinal divisions giving rise to sixteen or more spermatogenous cells (Fig. 5). Subsequent divisions of the fertile cells soon follow producing a mass of cells with large, distinct nuclei. The

divisions of the spermatogenous cells appeared to be generally simultaneous, although divisions have been noted occurring in one-half of an antheridium before the other (Stokey & Atkinson, 1956).

As the sperm cells develop, the nuclei begin to flatten and the protoplasmic material in the cell becomes less dense, membranes delineating the developing sperm now appearing quite distinct (Fig. 6). The mature sperms appear as flattened, coiled structures, usually occupying the central portion of the cell (Fig. 7).

The mature antheridium containing the coiled sperm is a rather large structure (Figs. 8, 17), protruding above the tissue of the prothallus. At times, the initial divisions in the developing antheridia contribute to the formation of a stalk upon which the antheridia are produced (Fig. 8). Considerable variation in the size and the visible pattern of organization of the mature antheridia was found to exist in cultured gametophytes. However, for the most part, both large and small antheridia on opening produced functional sperms.

In nature, archegonia are usually produced on the shaded or lower surface of the prothallus, along the sides of the midrib with their necks oriented almost at right angles to the thallus. However, when grown in culture, it is common for prothalli to have archegonia develop on both dorsal and ventral surfaces. As a result of the limited surface available in tubes, prothalli commonly grown on nutrient agar overlie one another in the course of their development. This overlapping

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FIGS. 1-8 — Stages in the development of the antheridium as seen in l.s. × 445·5. All structures are oriented as found in the natural condition. Fig. 1. Beginning of antheridial development evidenced by protrusion of the marginal cell and a periclinal division. Fig. 2. Second division (indicated by arrow) intersecting the first formed wall. Fig. 3. Two oblique anticlinal divisions have produced a wedge-shaped cell. Fig. 4. Formation of the primary spermatogenous cell (indicated by arrow) by the occurrence of a second periclinal division. Fig. 5. Synchronized divisions occurring in a regular pattern have produced a rather large structure containing many nuclei. Note the complex arrangement of the outer covering cells produced as a result of rapid divisions in these sterile cells. Fig. 6. Early stage in antheridial maturation. Nuclei have flattened and cell membranes are quite distinct. Fig. 7. Mature, coiled sperms each contained within its limiting membrane. Fig. 8. Large antheridium, containing mature sperms, which has formed on a slender stalk. Variation was seen to exist in the size of mature antheridia as seen by comparing Figs. 7 and 8.
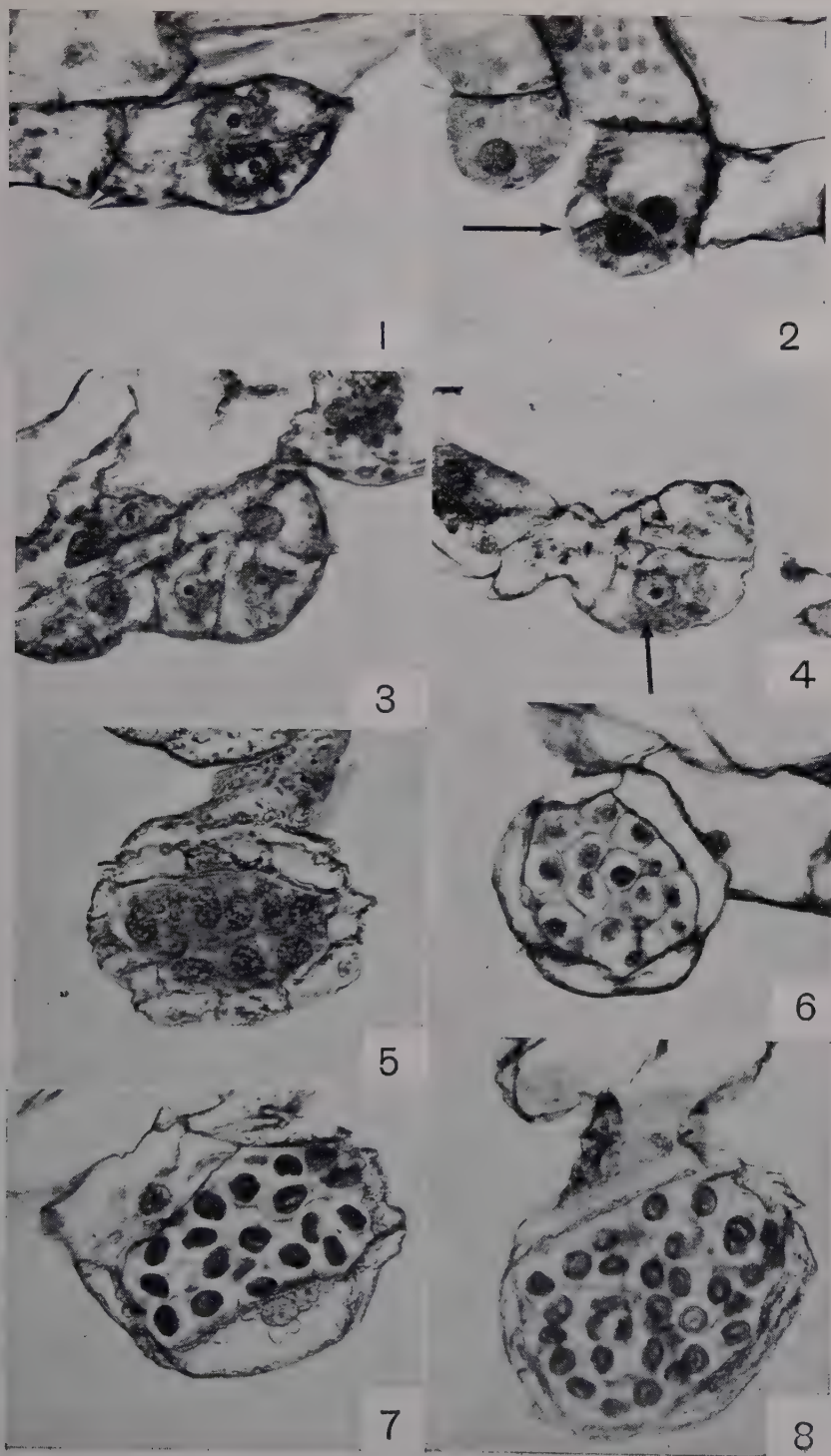
growth causes the dorsal surface to be shielded from light, and so produces the shaded condition seemingly favoring the initiation and development of archegonia on both surfaces. The majority of prothalli studied bore archegonia only on the sides of the midrib arranged in a series of irregular rows extending from just back of the apex of the prothallus to a point near the distal end of the thallus. By contrast, rhizoids were commonly produced over the entire central part of the cushion. It was not unusual to find in cultured prothalli archegonia which had their necks vertically oriented, developing from the central part of the cushion. The arrangement and position of the archegonia developed on cultured gametophytes of *Todea barbara* do not differ markedly from that of other members of the family (Stokey & Atkinson, 1956), although the occurrence of vertically oriented archegonia on the surface of the cushion appears to be more frequent in culture than in nature.
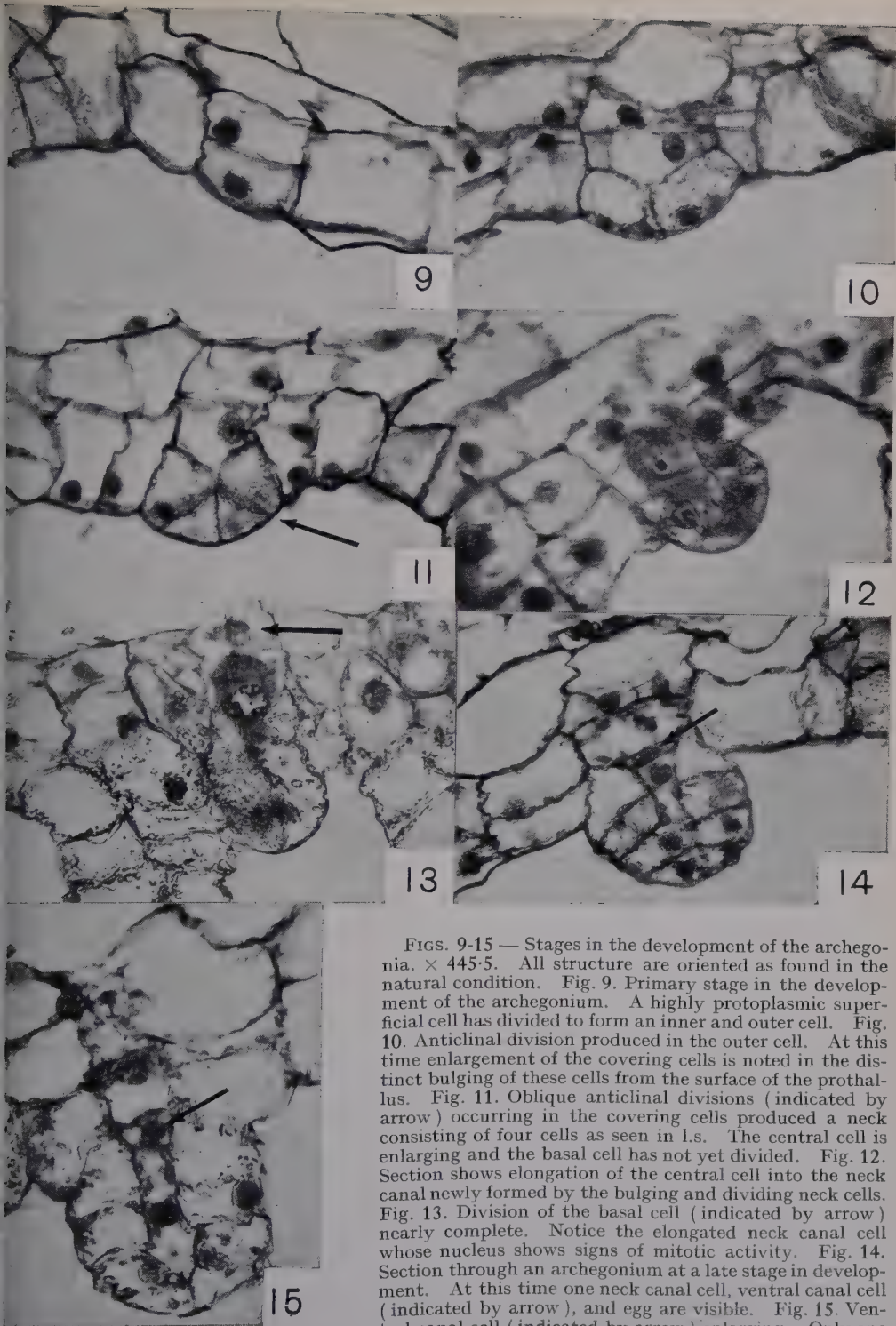
Of the many stages involved in the development of the archegonia, the most difficult one to recognize is the archegonial initial. Unlike its counterpart, the antheridial initial, which is characterized by a distinct bulging of the mother cell, the archegonial initial does not display any essential change either in size or shape of the cell. This is also true of other species of the Osmundaceae. When an archegonial initial is formed in *Todea barbara*, it can often be distinguished from the neighbouring superficial cells by its increased protoplasmic content.

During the development of the archegonia, the superficial initial first divides by a periclinal wall to form an outer and inner cell (Fig. 9). The inner cell then divides by a wall parallel to the first to form two cells. The archegonia at this stage consist of a row of three cells from which the axial row will be derived. The lowest will give rise to the bottom or wall cells of the venter; the middle cell will produce the neck canal cells, the ventral canal cell, and the egg; and the outer cell will produce the protruding neck of the archegonium. The next division to take place is an anticlinal one in the outer cell (Fig. 10).

The neck cells which up to this point had been parallel with the other prothallial cells now begin to grow and bulge upward from the surface of the gametophyte. Each neck cell then undergoes an oblique anticlinal division initiating a neck of four cells as seen in cross-section (Fig. 11). Simultaneous with the division and bulging of the neck cells, the central cell begins to elongate and fills the newly created neck canal of the developing archegonium. At this time, the neck consists of two tiers of four cells each of which has projected considerably from the basal prothallus (Fig. 12). The central cell is highly protoplasmic with only one nucleus and the basal cell has not yet divided.

Before the cells of the neck divide again, the basal cell is divided by an anticlinal wall (Fig. 13). At about the same time the central cell, which has been protruding into the canal formed by the developing neck, begins to divide (Fig. 13). The periclinal wall formed as a result of this division separates a neck canal cell from its larger sister cell. The latter again divides transversely to form the ventral canal cell and the egg cell. Figure 14 shows the appearance of an archegonium shortly after the ventral canal cell has been formed. At first, this cell is very small but as elongation in the neck takes place causing it to increase in length, the ventral canal cell enlarges and occupies a considerable area (Fig. 15). As the neck elongates, the single neck canal nucleus divides, commonly without wall formation, although the formation of a wall separating the two neck canal nuclei has been reported (Stokey & Atkinson, 1956). It was not seen in our material.

At maturity the archegonium is composed of six to eight tiers of neck cells, each tier made up of four cells, a long densely protoplasmic neck canal with two nuclei, a ventral canal cell which occupies the space formed by the lowest neck cells, and a somewhat flattened egg (Fig. 16). The egg cell in the mature archegonium contains a rather large centrally located nucleus embedded in a densely protoplasmic matrix. The protoplasm of the egg cell appears to be surrounded on all sides by a thin membrane depressed at the top by the mutual turgor pressures exerted

FIGS. 9-15 — Stages in the development of the archegonia. × 445·5. All structure are oriented as found in the natural condition. Fig. 9. Primary stage in the development of the archegonium. A highly protoplasmic superficial cell has divided to form an inner and outer cell. Fig. 10. Anticlinal division produced in the outer cell. At this time enlargement of the covering cells is noted in the distinct bulging of these cells from the surface of the prothallus. Fig. 11. Oblique anticlinal divisions (indicated by arrow) occurring in the covering cells produced a neck consisting of four cells as seen in l.s. The central cell is enlarging and the basal cell has not yet divided. Fig. 12. Section shows elongation of the central cell into the neck canal newly formed by the bulging and dividing neck cells. Fig. 13. Division of the basal cell (indicated by arrow) nearly complete. Notice the elongated neck canal cell whose nucleus shows signs of mitotic activity. Fig. 14. Section through an archegonium at a late stage in development. At this time one neck canal cell, ventral canal cell (indicated by arrow), and egg are visible. Fig. 15. Ventral canal cell (indicated by arrow) enlarging. Only one neck canal nucleus is present at this stage.

FIGS. 16-17 — Median longitudinal sections of mature sex organs enlarged to show structural detail. × 500. Fig. 16. Median section of a mature archegonium showing the long, densely protoplasmic, binucleated neck canal cell; the ventral canal cell; and the depressed egg with a large centrally located nucleus embedded in the protoplasmic matrix. Fig. 17. Median section of a mature antheridium containing numerous coiled sperms, each surrounded by a prominent membrane. The outer covering cells of the antheridium indicate the complex arrangement of this sterile tissue.

by the egg and the ventral canal cell (Fig. 16).

DEHISCENCE OF SEX ORGANS AND FERTILIZATION — Cultured gametophytes, grown on a fairly moist nutrient agar (0·8 per cent), provided an abundance of both large and small antheridia suitable for observing the opening of the antheridia and the subsequent discharge of sperms.

The first sight of the beginning of antheridial dehiscence can be noted a few minutes after the gametophytes have been placed in water. At that time, an increased swelling in the cells of the complicated, curved walls of the antheridium can be .detected. Associated with the swelling of the wall cells is the gradually increased size in the spermatogenous mass, evidence of the entry of water into the central cells of the antheridium (Hartman, 1931). The swelling of the wall cells occurs not only at the top of the antheridium, where generally the opercular cell will be evident, but also along the sides (Fig. 18). In most cases, the swelling proceeds slowly, the walls bulging distinctly until the opercular cell (in this species any wall cell of the antheridium) opens. The average time for this opening to occur in *Todea barbara* was fifteen minutes, although variations from five minutes to one hour have been observed for mature antheridia. The opercular cell in *Todea* may occur either on the side or top of the antheridium, the delicate and varied wall pattern making any premature designation exceedingly difficult. At dehiscence, the opercular cell is not completely disjoined but remains attached to the antheridial wall, in contrast to other reports (Hartman, 1931). No case was observed where the opercular cell was completely disjoined from the antheridia.

Opening of the opercular cell is quickly followed by the successive and rapid ejection of a number of coiled, motionless sperms. These sperms, still surrounded by mucilaginous material, remain stationary at the opening of the antheridium for six to ten seconds during which time the mucilaginous sheath is dissolved and the sperms slowly but steadily become active. They then begin a rapid, rotary motion and quickly swim away in their characteristic corkscrew manner (Fig. 19).

After almost half of the sperms have been ejected, succeeding sperms, as they leave the opening, exhibit a slight rotary

motion which continues for one to three seconds while the sperms are in the vicinity of the opened antheridium. Their motion increases rapidly whereupon they quickly leave the field of view. Many of the remaining sperms begin activity while still in the antheridium, their surrounding mucilaginous sheaths having been dissolved, at least in part, by the influx of water by way of the open opercular cell. These sperms continue their motion in the antheridia and, in contrast to sperms released earlier, are not ejected by the force of the antheridium, but swim out under their own energy.

Some variation in this description was noted in the dehiscence of many of the smaller antheridia. Even though these antheridia which contained fewer than fifty sperms opened in normal time, approximately fifteen minutes for *Todea barbara*, as stated, the mass of sperms they discharged were decidedly subnormal as evidenced by their prolonged inactivity.

In all, the empty antheridium is characterized by a swollen and irregular wall design resulting from the swollen nature of cells, both unequal in size and uneven in shape and by the presence of the opercular cell close to the antheridium (Fig. 20).

No correlation could be observed between the time of opening and antheridial size. Certain large antheridia containing many sperms (over one hundred) were noted opening before small antheridia, and, conversely, small antheridia on occasion opened before large ones.

In general, the timing of dehiscence of mature archegonia on cultured gametophytes of *Todea barbara* is variable. Mature archegonia usually open after immersion in water for twenty to thirty minutes, although a number of archegonia examined opened after two minutes in water and others not until after one hour of immersion.

Opening of the mature archegonia is first characterized by the gradual swelling of the four terminal cells of the neck (Fig. 21). Swelling begins immediately after the gametophytes have been placed in water and ordinarily continues for ten to fifteen minutes until the neck cells

separate leaving a central pore or opening. Prior to the opening of the archegonium, various changes begin to occur in the neck canal cells. These changes, evident by an increased capacity of these cells to take up certain stains, are probably associated with an increase in the amounts of hydrophilic material formed by the cells as they mature. An increased rate of cyclosis of the protoplasmic material in this region is soon noted and dehiscence is initiated by the movement of a mucilaginous mass of material from an opening, too small to be seen, between the four terminal cells. This movement, which in some cases occurs rather suddenly, is immediately followed by the extrusion of the protoplasmic material which can be seen flowing from the neck canal. Usually the protoplasmic material is ejected from the archegonium in a continuous, more or less, filamentous mass as described by Atkinson (1894), less often in *Todea* in the form of globules. Associated with the protoplasmic material, the two nuclei are often observed and in *Todea barbara*, these were the only globular entities eliminated from the neck canal. A sizable mass of this material is forced out of the archegonium before the terminal neck cells begin to part.

Once most of the neck canal content has been eliminated, the flow of mucilaginous material still proceeding, the four terminal cells of the neck begin to part in an orderly fashion. Along with the parting of the neck cells, or soon after these rows have assumed a more or less reflexed position, a globular body can be seen flowing from the archegonium. The elimination of this last mass in *Todea*, probably the ventral canal cell, marks the completion of the discharge of the axial row and the archegonium is now opened, an unobstructed passageway to the egg being available to the swimming sperm.

The discharge of the material occupying the neck canal and ventral canal cell in *Todea* is not forceful or explosive enough, in the case of cultured prothalli, to carry the exudate any distance from the archegonium, as has been reported for soil grown prothalli of *Polypodium* (Ward, 1954a). The expelled material moved

rather slowly out of the neck and remained adhering to one of the terminal neck cells. As the neck opened and the four rows of cells part, the exuded material is physically carried away from the mouth of the archegonium as a protoplasmic mass. The terminal neck cells of the archegonium were not thrown, but remain intact throughout the entire process of dehiscence. Dehiscence is usually completed in less than three minutes, although cases have been observed in *Todea barbara* where only partial disintegration of the axial row was observed and incomplete dehiscence occurred, portions of the canal material not being eliminated at all.

The process of fertilization in *Todea barbara* follows essentially the same pattern as has been described for other fern species (Campbell, 1892; Shaw, 1898; Atkinson, 1894; Conard, 1908; Mottier, 1904; Ward, 1954a). Liberated sperms swimming in the vicinity of the archegonium appear to be attracted by substances diffusing from the opened neck. Pfeffer (1884), Buller (1900), and more recently, Rothschild (1956) have demonstrated that many substances, most notably malic acid and its salts, have the ability to attract sperms of many ferns. This substance, or some related compound (Shibata, 1911), is considered to be secreted by the egg or the neck canal cells and, as a result, attracts the sperm by producing a gradient towards which the sperms swim. Sperms which have been chemotactically attracted approach the mouth of the archegonium where their movement becomes somewhat retarded. They tend to remain there for just a short time, usually not more than a minute, more commonly only a few seconds. They may then move away from the vicinity of the opened archegonium, their helical movement again accelerated behaving in much the same way as sperms of *Phlebodium aureum* (Ward, 1954a) and those of *Gymnogramma martensii* (Buller, 1900). After a few minutes, the sperms again begin to collect around the mouth of the open archegonium, their movement at this time not being visibly affected.

The sperms then quickly enter the opened neck of the archegonium and proceed towards the egg. As they approach the egg, they do not retain the coiled arrangement characteristic of this species, but become greatly elongate exhibiting a marked helical motion, more rapid than previously observed. Many sperms were seen to enter the canal of the opened archegonium, each becomes stretched and its rapid movement above the egg can be distinctly observed.

Although the neck canal appears wide enough in most cases for the sperm to enter without having to alter its coiled shape, no instance was observed in which entering sperms did not elongate. In fact, many sperms on entering the neck canal of the archegonium became almost straight assuming a shape similar to that described by Shaw (1898) and Conard (1908) for sperms of other species.

In many cases, the mass of spermatozoa in the canal of the archegonium was noted to retain activity for as long as one hour, during which time one of the sperms had successfully penetrated the egg membrane and effected fertilization.

The mature egg in *Todea barbara* (Fig. 16) does not round off after the axial row has been eliminated, but remains flattened until after fertilization is effected, in contrast to the observations of Shaw (1898) and Conard (1908). An embryo which had not been fertilized and still exhibits the depressed, flattened character of the egg in an opened archegonium is shown in Fig. 22.

Once the egg has been fertilized, the distal cells of the neck come together, the thin membrane which has surrounded the egg from its inception becomes turgid and the whole egg expands, filling the space previously occupied by part of the ventral cell (Fig. 23). The recently fertilized egg, now spherical, consists of an upper vacuolate region and a lower densely protoplasmic region containing the nucleus. At times, the egg arches up into the canal with such turgidity that it assumes the shape of the venter and in many cases acquires a pointed upper surface (Fig. 24) into the neck canal as has been observed for embryos of other species by Atkinson and by Conard.

Fertilization, as it occurs in *Todea barbara*, is an extremely efficient and successful process. Many gametophytes
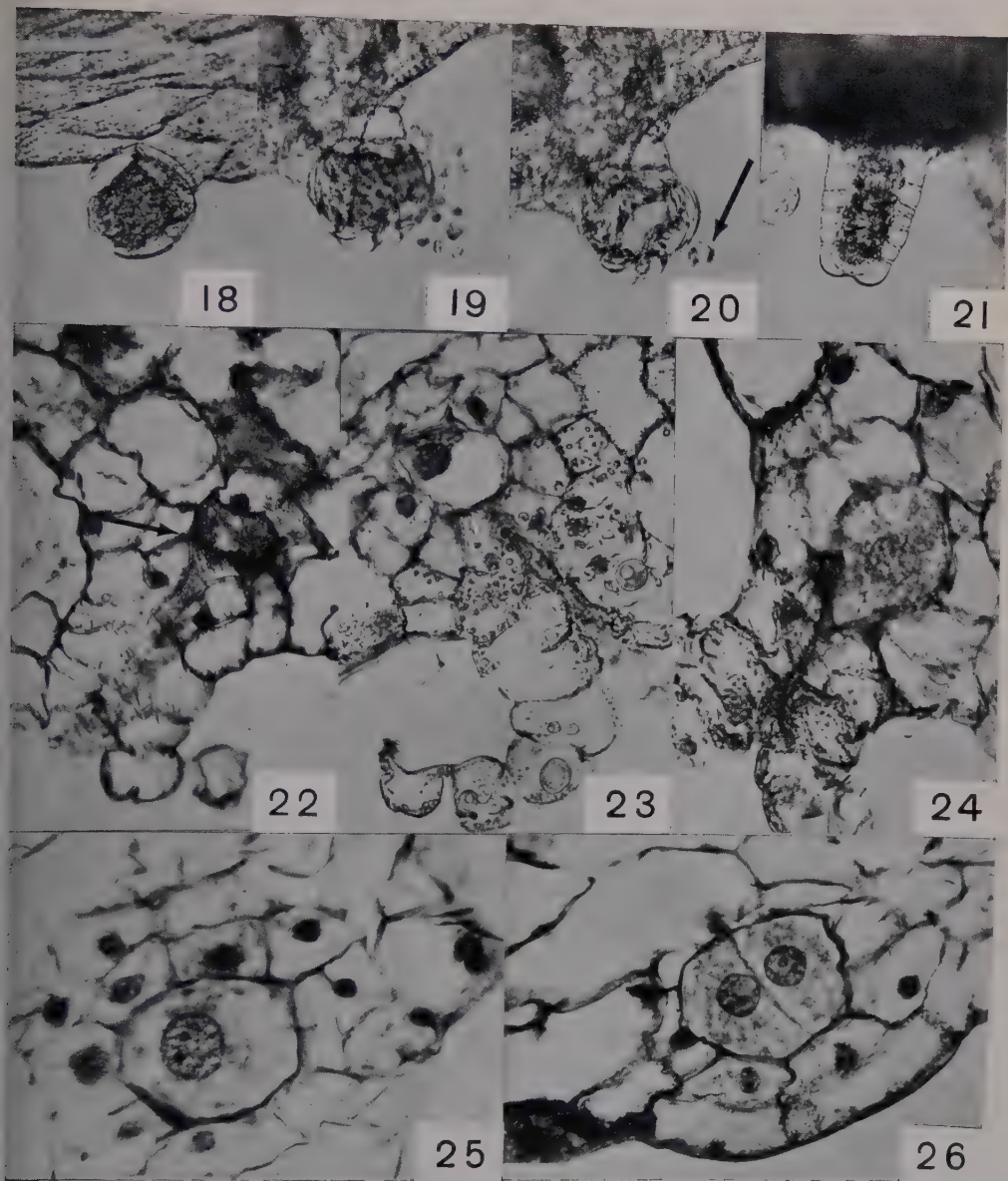
Figs. 18-26 — Figs. 18-21. Dehiscence of sex organs. × 202·5. Fig. 18. Mature antheridium in water beginning to swell. Fig. 19. Antheridium discharging sperms. Fig. 20. Empty antheridium showing the complicated pattern of the wall. Opercular cell attached (indicated by arrow). Fig. 21. Mature archegonium in water displaying the first signs of dehiscence. Figs. 22-24. Median section through archegonia bearing unfertilized and fertilized eggs. × 324. Figs. 22. Depressed condition of egg (indicated by arrow) visible in l.s. of opened archegonium. Fig. 23. Vacuolate condition of turgid embryo evident in l.s. of opened archegonium. Fig. 24. L.s. of an archegonium in which the fertilized egg has expanded into the neck canal cell. Figs. 25-26. Sections of young embryos in the early stages of development. × 405. Sections are parallel to the prothallial axis and at right angles to the longitudinal axis of the archegonium. Fig. 25. Undivided embryo 5 days of age surrounded by dividing calyptra cells. Fig. 26. 6-day old embryo with first division completed.

which had been fertilized in the usual manner and placed on a nutrient agar were found after twenty-four to forty-eight hours bearing numerous growing embryos. (A similar condition was not noted for fertilized prothalli placed on soil to develop.) It was not unusual, on cultured gametophytes, to find as many as fifteen growing embryos on the same prothallus in many different stages of development. Fertilization appears to be almost 100 per cent effective in this species and very rarely did a gametophyte possessing mature sex organs fail to produce at least one growing embryo, in marked contrast to reported results of other ferns (Hofmeister, 1851; Atkinson, 1894; Conard, 1908; Hoyt, 1910; Ward, 1954a). None of these embryos was ever seen to be apogamous, all having their origin in archegonia.

EMBRYOGENY — The young embryo of *Todea barbara* lies turgid in the venter of the archegonium surrounded by a layer of highly protoplasmic, dividing, calyptra cells possessing conspicuous nuclei (Fig. 25). In the early stages of its development the embryo is in close contact with the jacket cells of the venter, the membrane surrounding the embryo being tightly appressed to these cells. No definite proofs of physical attachment in the prospective foot region were found, as has been reported for the embryo of *Polypodium aureum* (Ward & Wetmore, 1954), in embryos of *Todea* until a much later time when development had progressed to a point where differentiation had already begun and the foot region was in intimate contact with the parent thallus. This is somewhat amazing inasmuch as these cells must have been in continuity in the axial row. When this continuity was lost, and how the wall separation took place is not recognized as yet, despite considerable work on the problem.

Characteristically, the shape of the young embryo of *Todea* is not rigidly fixed. It may be round, oblong, or elongated ultimately assuming the shape of the cavity within which it develops. As the embryos grow, prior to the first division, many elongate in the direction of the archegonial axis before any nuclear activity is detected. Nuclear activity in

embryos of *Todea barbara* is usually evident, in sectioned material, five days after the fertilization period. At this time the enlarging nuclei of many embryos observed were in various preparatory stages of division.

The first division of the embryo on cultured gametophytes occurs within the sixth day after fertilization (Fig. 26). The position of the first wall formed as a result of this division is not as regular nor as uniform as has been reported for other leptosporangiate ferns (Vladesco, 1935; Campbell, 1892; Ward, 1954b; Wardlaw, 1955). Commonly, the first division of the zygote occurs parallel to the long axis of the archegonium and at right angles to the anterior-posterior axis of the prothallus. However, many instances were observed in which the first wall formed at an angle of forty-five degrees and less to the longitudinal axis of the prothallus and in some cases closely approached being parallel to it. On examining a large number of these embryos, it was found that the archegonia within which the embryos formed were arranged obliquely at varying angles to the long axis of the thallus instead of perpendicular to it. Whether this is in some way related to a more ready fertilization in the nearly horizontal archegonia in water available is not clear as yet. More work has yet to be done. The first division regularly formed parallel to the longitudinal axis of the archegonial neck, and in the case of obliquely oriented archegonia, the first wall was laid down at an angle to the anterior-posterior axis of the prothallus. A careful survey of the plane occupied by the first formed wall appears to point out the importance of the orientation of the archegonial axis in determining the position of the first wall.

According to classical accounts of fern embryology (Hofmeister, 1851; Vladesco, 1935), the first wall separates the " epibasal " or anterior portion, which gives rise to the shoot and first leaf, from the " hypobasal " or posterior portion which is destined to form the foot and first root. Succeeding divisions follow, the first few occurring slowly (a fifteen-day old embryo of *Todea* being composed of ca. sixty-four cells) until a sizable aggregation of

cells is attained, whereupon divisions ensue rapidly until the subsequent formation within of a group of procambial elements after which and only after which are differentiated regions (organs) formed: leaf, root, stem apex.

Preceding the growth of the embryo, the jacket cells of the archegonium divide periclinally forming an enveloping layer or calyptra, two, and in many cases, three cells thick, which completely surrounds the growing embryo. The embryo of *Todea*, like the embryo of *Osmunda* (Cross, 1931; Campbell, 1892), retains its globular appearance and develops within the confines of the calyptra for a longer period of time than the more advanced ferns. In *Todea barbara*, the embryo remains within the calyptra for about thirty days after it has been fertilized, by which time the developing organs (root and shoot) are rupturing the jacket of surrounding calyptra and soon the young sporophyte is established as an independent and self-sufficient plant.

Discussion

Prothalli of *Todea barbara* growing on a nutrient medium exhibit to a much smaller degree the various morphological expressions of growth which characterize so many other fern species. Steeves *et al.* (1955) found the developmental pattern of cultured gametophytes of the bracken fern, *Pteridium aquilinum*, differed to a large extent from those grown on soil. Aside from certain abnormal proliferations present on cultured gametophytes, the most noticeable difference was found in the prolongation of the filamentous stage of prothallial growth. The same type of growth response has been demonstrated by Hurel-Py (1950) for a large number of gametophytes of both the Polypodiaceae and Cyatheaceae grown in culture. These writers agree that prothalli, grown in sterile culture, are not typical for prothalli growing in nature since their developmental pattern differs from that of soil grown prothalli. The development of cultured gametophytes of *Todea barbara* is not characterized by any extended period of filamentous growth, but proceeds along pathways

which diverge only slightly from those of naturally growing plants. The most pronounced feature observed in cultured gametophytes of *Todea* is the prolonged growth of the thallus and the great prodigality of numerous prothallial outgrowths from both the wings and midrib region when the apical region of the prothallus ceases to function.

The effect of environmental factors on the development of gametophytes is well documented (Prantl, 1879; Williams, 1938; Mohr, 1956). Recently, cultural experiments have demonstrated that development can also be controlled by manipulating the content and concentration of various growth factors (Hurel-Py, 1955; Sossountzov, 1953; Steeves *et al.*, 1955).

Cultured gametophytes of *Todea*, as they develop to maturity, exhibit a pattern of growth which corresponds closely to that observed for soil-grown prothalli by Stokey & Atkinson (1956) and Luerssen (1874). In this respect, it is pertinent to note that the growth of gametophytes of *Phlebodium aureum* on soil was observed by Ward (1954a) to parallel that of agar-grown plants, although those grown on soil were noted to attain maturity slightly before those grown on agar. Gottlieb (1958) has illustrated a similar growth response for gametophytes of *Pteridium aquilinum*.

The sex organs on cultured gametophytes of *Todea barbara* originate in their normal sequence in ferns, antheridia appearing before archegonia. Although in nature the prothalli characteristically bear antheridia and archegonia only on the lower or ventral surface, in culture many gametophytes develop sex organs on both surfaces. A number of experiments previously performed (Heinricher, 1888; Prantl, 1881; Williams, 1938) have indicated that the position of sex organs is determined by the illumination which the gametophyte receives. When gametophytes of *Todea* are grown isolated and are illuminated from above, sex organs appear only on the lower surface as is usually the case. In culture, however, gametophytes of *Todea* grow randomly and, aside from overlying one another in their congested growth, may be oriented

in various positions. Some grow normally, lower surface down, and sex organs develop in the usual position; others, however, may be arranged with their lower or ventral surfaces up and are illuminated, in which case, sex organs form on the upper or dorsal side. Still others may grow vertically, sex organs originating on both surfaces of the thallus.

Studies by Prantl (1881) have shown that gametophytes of *Phlebodium aureum* developed archegonia on both sides of the prothallus when grown in weak light. Information is not presently available which would enable us to determine whether the position of. the sex organs is affected by the quality or the intensity of the illuminating source. Further, it is not yet clear what part humidity plays in these observations.

Recent investigations of Naf (1956, 1958) have demonstrated that the formation of antheridia in various members of the Polypodiaceae, Dicksoniaceae, and Schizaeaceae, is due to the elaboration of an " antheridial inducing factor " by the prothallus. It seems quite possible, in light of this evidence, to postulate that the formation of sex organs and the positions they occupy on the gametophyte may be governed and controlled by two entirely different sets of factors, an internal factor controlling formation and an external, controlling position.

Mature antheridia of *Todea* are formed by a series of divisions quite unlike those described by Davie (1951) for the higher leptosporangiate ferns. The nature of the divisions and the large size attained are indicative of the primitive character of the antheridium whose structure closely resembles antheridia of species from both the Matoniaceae and Gleicheniaceae (Stokey & Atkinson, 1950, 1952).

A number of abnormalities in the archegonia of soil grown gametophytes of *Todea barbara* have been recorded. Stokey & Atkinson (1956) observed archegonia which exhibited a cross wall in the neck canal cell and the presence of two or three ventral canal cells. Other archegonia, having two ventral canal cells, were observed by these workers, as was the phenomenon of " twinning ", in which two

archegonia are formed from adjacent cells and their eggs share a common calyptra. Luerssen (1874) noted, in his material of *Todea barbara*, that the neck canal cell regularly possessed only one nucleus.

As observed in this study, the only abnormality displayed by archegonia of *Todea* was that of " twinning " which was frequently observed in cultured gametophytes. At times, two or more pairs of twin embryos appeared on a single plant. The presence of a cross wall in the neck canal cell and the appearance of multiple ventral canal cells were not observed in this study. All of the hundreds of archegonia examined were uniform in appearance and possessed one neck canal cell with two distinct nuclei, one ventral canal cell, and the egg.

Previous investigations of antheridial dehiscence have been carried out almost exclusively on members of the higher ferns, the Polypodiaceae being a frequent choice of material. The accounts of Schlumberger (1911), Hartman (1931), and more recently, Ward (1954a), are among the most complete. The writers generally agree that the opening of the mature antheridium is almost instantaneous and followed quickly by the forceful ejection of the first group of sperms which remain motionless for a short time before beginning activity.

The type of dehiscence observed for antheridia of *Todea barbara* differs somewhat from the reports presented. Although antheridia were observed to open suddenly in water, the ejection of sperms was not found to be as prolonged nor as effective in completely eliminating the sperm as reported for the more advanced ferns. Only a portion of the numerous sperms contained in the mature antheridia are forcefully discharged, the remainder leaving the antheridium under their own power, some slowly and others very quickly.

The variation in dehiscence reported may possibly be due to a structural difference in the antheridia of the two families, those of the Polypodiaceae being structurally " simpler " than that of the Osmundaceae. Hartman observed that the inward protrusion of the distended peripheral cells of the antheridium was

instrumental in ejecting the sperms and Atkinson observed that the action of the superficial cells of the antheridium assures the expulsion of the sperm. In *Todea*, there are no single peripheral cells making up the antheridium, but the antheridium wall is composed of a series of variously arranged and connected curved cells. The complexity of the antheridium in *Todea* makes it appear improbable that any few cells serve effectively to force out a large number of sperms, although a similar system may be temporarily active and account for the forceful elimination of some sperms.

Experiments of Ward using prothalli of *Phlebodium aureum* grown on a nutrient agar demonstrated that sperms were released from the antheridia "under considerable internal pressure". It does not seem likely, therefore, that the variations noted in this study are due entirely to the existing cultural conditions under which the prothalli were grown.

The opening of the archegonia in *Todea* is not associated with any forceful expulsion of the canal cells as observed by Ward for soil grown prothalli of *Phlebodium*. Movement of the protoplasmic material in the canal cells is obvious, in many cases, even before the archegonium opens. The rather stringy protoplasmic material flows slowly out of the archegonium and remains near the entrance until the bending of the vertical rows of neck cells carries it away. Atkinson also noted that the canal cells protruded from the archegonia in the form of a slimy, granular stringy mass.

The appearance of the egg cell of many ferns, immediately after the canal cells have been eliminated and after fertilization has been effected, has been described somewhat differently by different workers. Atkinson (1894) records the egg cell of many ferns to be concave at the top until after fertilization takes place when the fertilized egg increases in size and completely fills the space in the base of the archegonium. Campbell (1892) observed the egg of *Osmunda* to be flattened and upon being fertilized acquired a membrane and assumed a rounded shape. The concavity of the egg of *Onoclea sensibilis* before fertilization was seen by Shaw

(1898) who also noted that the egg becomes swollen and turgid as soon as the canal was cleared. According to Conard (1908), the embryo of the hay-scented fern, *Dennstaedtia puntilobula*, is flattened until the canal is emptied, at which time it swells and becomes pointed. Yamanouchi (1908) found the egg cell of *Nephrodium molle*, prior to fertilization, to be flattened and the protoplast exposed, the dome-shaped cell wall which separated the egg from the ventral canal cell having disappeared. No further mention is made by Yamanouchi of the acquisition of a membrane around the egg, or the appearance of the egg after fertilization, although his figures show the fertilized egg to be swollen and turgid. Mottier (1904) observed that the egg cell of *Onoclea struthiopteris* is concave on the upper surface until the archegonium opens, then it swells and fills the venter. He also casts some doubt on Shaw's (1898) observation of a collapsed condition of the egg after the entrance of the sperm.

In *Todea barbara*, the mature egg is similar to that of other species in being concave on its upper surface, but, in contrast to previous reports using other ferns, it is completely surrounded by a thin transparent membrane. After the opening of the archegonium, the egg cell of *Todea* does not swell or become turgid. Sectioned material shows the egg to remain concave until the penetration of a spermatozoid was affected. At this stage, in *Todea*, and not until the process of fertilization is initiated, does the embryo become turgid and assume a spherical or pointed shape.

Atkinson (1894), Shaw (1898), Conard (1908), Hoyt (1910) and Ward (1954a) are among those who have reported for their respective material, a large majority of apparently normal gametophytes with mature sex organs which did not produce embryos. These investigators, for the most part, utilized soil grown prothalli in their experiments. After fertilization the prothalli were placed on soil or peat and examined at various intervals for the presence of embryos. In cultures of *Todea barbara*, which were fertilized and allowed to develop on soil, a similar phenomenon was observed. When the prothalli were

planted on a nutrient medium after fertilization, each bore at least one, and in the majority of cases, several functional embryos, one of which developed to the mature sporophyte.

In *Todea* during early embryonic growth, before and after the first division, embryos were easily removed uninjured from their position in the venter. The presence of embryos of *Todea barbara*, not obviously attached to the venter wall, was the greatest morphological variation observed in this study. No reference could be found of the occurrence of a similar situation among other ferns.

The first division in the embryo of *Todea* occurs parallel to the longitudinal axis of the archegonial neck. The position of this wall, in relation to the prothallus, was found to vary depending on the orientation of the archegonia. Wardlaw (1955), reviewing the experiments of Leitgeb (1878, 1880), performed on *Marsilea* megaspores, calls attention to the importance of the " zygote archegonium complex " as one of the master factors in embryonic development. Leitgeb's results demonstrated that the first wall produced in the megaspores of *Marsilea* could not be induced to form in any plane other than that of the archegonial axis. In *Todea* it appears that this is also the case, the position of the first wall being determined by the orientation of the archegonium. As the archegonia are usually oriented almost horizontally, growing at right angles to the midrib, the first division would occur at right angles to the anterior-posterior axis of the prothallus as is usually the case for the higher leptosporangiate ferns. If the archegonium is oriented obliquely on the thallus, the first division would be parallel to the axis of the archegonia and at a variable angle to the longitudinal axis of the gametophyte. Campbell (1892) makes no mention of any variability in the embryo of *Osmunda*, although Cross (1931) notes the first division to form at an angle to the longitudinal axis of the gametophyte.

Summary

An examination of the various stages in the life history of *Todea barbara*, as they occur in sterile culture, beginning with the germination of spores and culminating in the production of the sporophytic plant, has demonstrated the remarkable stability of this species. It proved able to respond to all changing environmental conditions of culture, both nutritional and physical, to which it was subjected during these studies without harmful effects on growth. Moreover, these changes were accomplished without altering significantly either the timing or sequence of the various reproductive stages from those commonly occurring in nature.

Considerable variation was observed when certain stages in the life history of *Todea* were compared with those of the " higher " ferns, as exemplified by members of the Polypodiaceae. Aside from the obvious morphological differences between the adult or sporophytic generations, major differences were noted in the formation, position, and dehiscence of sex organs. These differences, significant from a phylogenetic viewpoint, indicate that the various stages in the life history of a fern, characterized over the years by the " higher " ferns, are not as fixed and rigid as was once thought. In view of the results presented here, it is suggested that any introduction or study concerned with the life history of a fern, or the various component stages, be approached with a reasonable amount of flexibility, always cognizant of the existing variations.

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MORPHOGENETIC STUDIES ON THE FERN *TODEA BARBARA* (L.) MOORE — II. DEVELOPMENT OF THE EMBRYO

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Introduction

Since the early embryological investigations of Hofmeister (1851), great volumes have been added to our knowledge of the embryology of both the higher and lower plants (*see* reviews by Bower, 1923 ; Campbell, 1918 ; Eames, 1936 ; Wardlaw, 1955 ; Johansen, 1950 ; Maheshwari, 1950 ; Foster & Gifford, 1959). However, contributions to the field of fern embryology have, for the most part, been of a descriptive and histological nature. Up to the present, few investigations in the field of experimental morphogenesis, genetics, and biochemistry of the developing plant embryo have been carried out. In view of the increasing amount of research devoted to problems of plant growth and morphcgenesis (*see* review by Wetmore & Wardlaw, 1951), it might seem surprising that little attention has been given to the study of the developing fertilized egg in plants. However, it can be understood that the reason behind this stems from the relative inaccessibility of the egg before, during and after fertilization.

A study of the developmental stages in the life history of *Todea barbara* has shown that the embryo of this species, in its early stages, is not physically attached to the walls of the venter and, by the use of suitable manipulative techniques, can be removed uninjured from the prothallial environment within which it normally develops. The fact that these young embryos could be removed in numbers from their position within the archegonium and successfully grown in sterile culture suggested the possibility of their use in studies designed to determine the relation of containment as a morphogenetic influence upon the planes of early cell division and subsequent development of the fertilized embryo. Before these studies could be undertaken, it was necessary to investigate in detail the various stages in the growth and development of the fertilized embryo in its natural environment, contained within the venter, in order to make available a descriptive account of the usual sequence of events which takes place in the growing embryo under natural conditions. The embryological study of

Todea barbara, presented in this paper, would thus serve as a foundation and basis of comparison for the experimental studies to follow.

Some attention has already been given to the study of the developing embryo of the Osmundaceae. Earlier workers attempted to correlate the embryological features of this group with other morphological characteristics in order to determine the relationship of the Osmundaceae to other families of ferns and assign to it a phylogenetic position (e.g. Bower, 1923).

Campbell (1892), in his comprehensive study of *Osmunda claytonia* and *Osmunda cinnamomea*, found the embryological data supported other morphological features which indicated that this group of primitive ferns is more closely allied to the leptosporangiate than to the eusporangiate ferns.

Cross (1931), reviewing the embryology of *Osmunda cinnamomea*, agrees that the Osmundaceae may be intermediate between the eusporangiate and the leptosporangiate ferns with respect to embryological features. However, he finds the data from his studies to support the view that *Osmunda* stands nearer the eusporangiate than the leptosporangiate level.

Up to the present time, information concerning embryo development in other genera of the Osmundaceae has been lacking and no comparison of embryo development on related genera has been made which would support or refute the interpretations which have, for this group, been based solely on the study of the embryo of *Osmunda*.

A detailed embryological study of *Todea barbara* is, therefore, considered necessary, not only to establish the usual developmental sequence in embryonic development on which further experimental studies can be based, but also for the value such an embryological record may have for future taxonomic and phylogenetic considerations.

The present study considers the various stages through which the fertilized egg of *Todea barbara* passes as it develops from the initial unicellular condition to the point at which, through repeated divisions, it becomes multicellular and develops conspicuous appendages. Considerable

attention has been given to determining not only the sequence of organ formation, but also the exact time of their initiation.

Materials and Methods

Prothalli of *Todea barbara* (L.) Moore utilized in this portion of the investigation were grown in sterile culture and fertilized according to the procedure reported in a previous communication (DeMaggio, 1961). After fertilization, the prothalli were transplanted to Petri dishes containing a modified Knudson's medium (Steeves *et al.*, 1955), with 1 per cent sucrose and allowed to develop under controlled conditions of light and temperature. Methods used in examining prothalli for various stages of embryo development are given in detail in the first paper in this series.

The determination of the sequence of events occurring in embryological development, particularly in the early stages, was significantly enhanced by utilizing excised embryos in addition to fixed and sectioned prothallial material.

Excised embryos were examined directly under the microscope as whole mounts, without benefit of any stain. This technique of examination supplemented measurably the examination of sectioned material and presented an effective method of visualizing the growing embryo in its natural condition as a three-dimensional structure.

Observations and Results

TERMINOLOGY — The descriptive terms used to designate the orientation and sequence of the early divisions in the young embryo of leptosporangiate ferns has not met with any general approval by the various workers in this field.

It is generally accepted that the first partition wall in the fertilized embryo of most leptosporangiate ferns occurs in the long axis of the archegonia and at right angles to the anterior-posterior axis of the prothallus. This division separates the embryo into an anterior portion situated towards the prothallial apex or notch and a posterior portion directed away from the apex. Voux (1877) designated the primary division as the " basal " wall and

employed the terms " epibasal " and " hypobasal " for the anterior and posterior regions of the divided embryo.

The terms " epibasal " and " hypobasal" although used quite often in descriptive fern embryology (Wardlaw, 1955), are considerably more important in describing the separation of the two halves of the embryo in the eusporangiate ferns than in the leptosporangiate. In many eusporangiate ferns and in certain genera as *Psilotum*, *Tmesipteris*, and *Equisetum* (Bierhorst, 1953; Holloway, 1921; Campbell, 1928), the first division of the embryo actually separates two halves of the embryo, one above the other. In leptosporangiate ferns, however, these terms are, as Ward (1954) has pointed out, without significance since the two halves of the embryo produced after the primary divisions are not usually situated one above the other, *Cardiomanes reniforme* of the Hymenophyllaceae (Holloway, 1944), being a notable exception. For the mojority of the leptosporangiate ferns, the terms anterior and posterior are to be preferred, as these terms more accurately depict the true orientation of the two halves of the divided embryo to one another and to the prothallus, the anterior half being toward the apex or notch of the prothallus.

After the basal wall has formed, the next two walls to appear separate the embryo into quadrants and octants respectively. Although a number of earlier investigators were inclined to consider the orientation and sequence of these divisions as fixed and rigid, Voux (1877) found that in *Asplenium shephardi*, the second pair of divisions which segmented the embryo into quadrants was in some cases perpendicular to the basal wall and perpendicular to the axis of the archegonium and in other cases observed, was perpendicular to the basal wall but parallel to the axis of the archegonium, thus demonstrating that both the sequence and position of the second and third partition walls may vary.

The designations quadrant and octant walls are still useful terms in describing the sequence of partitioning of young embryos. However, recent investigations have indicated that the rigid concept of quadrants and octants held by earlier workers has been greatly overemphasized and, if these terms are employed, their use should be restricted to regional partitioning and no morphological significance should be given to them.

In this respect, the terms " transverse " wall and " median " wall, although originally coined by Voux (1877) to represent the quadrant and octant stages, may even be of greater value in modern day terminology since they actually depict the relative position of the division walls. It should be emphasized that the proper use of these terms at the present time is to designate only the position of the division walls which occur and no attempt should be made to imply a fixed sequence to the order of their appearance.

After the transverse walls are laid down, irrespective of whether they are formed by the quadrant or octant divisions, the embryo is segmented into superior and inferior as well as anterior and posterior hemispheres. The inferior hemisphere is designated as that portion of the embryo lying close to the neck of the archegonium on the naturally oriented prothallus, with the archegonia below, and the superior hemisphere, that portion of the embryo situated above it, that is, farthest from the neck of the archegonium. Employing the terminology of Vladesco (1935), once the octant stage has been attained, the main regions of the embryo may be conveniently designated as: anterior-superior, anterior-inferior, posterior-superior, and posterior-inferior, each consisting of two cells. Although these terms have been used in a general way to indicate various organographic regions, they are, nevertheless, purely descriptive and are intended only to point out topographical regions of the young embryo.

Recent investigators (Vladesco, 1935; Wardlaw, 1955; Ward, 1954) have asserted that to ascribe rigidly the formation of an organ to a particular descriptive region is a practice which in most cases should be abandoned. These workers have shown that the formation of various organs, particularly foot and stem, is not limited to any one morphological region, but may involve neighbouring regions.

The terminology used in the present study to describe the early pattern of segmentation in the young embryo of *Todea* is essentially that employed by Voux and Vladesco. In certain instances, the terms have been slightly modified in order to describe more accurately not only the position of the division walls but also the various regions of the growing embryo.

EARLY EMBRYOLOGY AND SEGMENTATION — The mature egg of *Todea barbara* resting in the venter of the archegonium has been observed to be completely surrounded by a thin transparent membrane. Sectioned material has shown that prior to fertilization the egg appears flattened on the surface in contact with the ventral canal cell, a condition which is characteristic for many leptosporangiate ferns (Atkinson, 1894; Campbell, 1892; Shaw, 1898; Conard, 1908; Yamanouchi, 1908; Mottier, 1904). Even at this stage in development, the egg cell contains an abundance of fully developed chloroplasts distributed rather evenly throughout the flattened egg.

Once fertilization has been successfully affected, the young embryo becomes turgid, the flattened surface arching up and completely filling the space in the archegonial venter. At this time, the young embryo is characterized by having an uneven distribution of chloroplasts, most of the cytoplasmic material containing the chloroplasts and nucleus being situated in that portion of the embryo farthest away from the neck of the archegonium. The portion of the embryo which has expanded after fertilization becomes highly vacuolated. Figure 1 shows the appearance of an embryo isolated from the archegonial venter four days after fertilization, the vacuolate condition in the recently expanded portion of the embryo being evident. Most embryos, four days after fertilization, were found to be between thirty-five and forty microns in diameter and anywhere from thirty-five to fifty-five microns in the axis of the archegonium. Embryos at this stage in development were noted to burst immediately on contact with water and to undergo rapid plasmolysis when immersed in sucrose solutions at a concentration above 0·1 molar. In most of the examinations carried out using excised embryos, they were removed from the prothallus in a 0·03 molar sucrose solution since this concentration seems essentially isosmotic in that it produced no visible evidence of plasmolysis or deplasmolysis.

From the fourth to the sixth day following fertilization, the young embryo exhibits signs of increased growth and, associated with this increase in size, various internal changes are visible. A number of embryos examined by excision shortly before the first division had occurred had increased to an average size of fifty-five microns in width and sixty-eight microns in length. Many of these embryos were still highly vacuolated, but others displayed signs of a more even distribution of chloroplasts and other cytoplasmic materials within the embryo (Fig. 2).

The primary division of the embryo was found to occur six days after fertilization. This first or basal wall is formed in the axis of the archegonia and at right angles to the anterior-posterior axis of the prothallus as is characteristic for most of the leptosporangiate ferns. However, it was noted that the position of this wall was not constant for all the embryos examined. Many embryos showed the basal wall to be inclined towards the apex of the thallus rather than at right angles to the long axis. This condition is not limited to embryos of *Todea* but has been noted to occur in other species of ferns as well (Cross, 1931; Vladesco, 1935). The first division separates the embryo into anterior and posterior hemispheres. Observations on excised embryos at this stage have indicated that the basal wall divides the embryo into two rather equal halves (Fig. 3). In their embryological studies, Vladesco (1935) and Atkinson (1894) noted that commonly the first division of the embryo in many species of ferns separates it into two unequal portions, the posterior portion being somewhat larger than the anterior. No evidence for the existence of a similar condition was found in embryos of *Todea*.

A few embryos which had undergone a primary division were found to retain still the vacuolate condition characteristic of undivided embryos, although these were comparatively few in number.

Following the formation of the basal wall, the nuclei in both anterior and posterior portions of the embryo begin to show signs of activity. In all cases examined, both nuclei were seen to undergo division simultaneously, partitioning the embryo into quadrants. These second or quadrant walls, which form in the embryo of *Todea barbara* seven days after fertilization, occur in the plane of the archegonium and generally parallel to the long axis of the thallus. At times this wall was noted to form at a variable angle but always transecting the basal wall at right angles. It is customary to speak of a wall in this plane as the median wall. At the time this division occurs, the average size of the embryos is sixty-eight microns in width and seventy-one microns in length, and the embryos appear to have attained a rather homogeneous distribution of cytoplasmic materials (Fig. 4).

Octants are formed characteristically in embryos of *Todea* nine days after fertilization. At this time a division occurs simultaneously in each of the embryo quadrants usually formed perpendicular to the axis of the archegonium and to the two previously formed walls. These transverse walls, as they are usually designated, succeed in segmenting the embryo into a superior and inferior portion, both hemispheres composed of two anterior and two posterior octants.

At this stage in development, most of the embryos examined by excision and subsequent embedding and sectioning displayed an average size of seventy-five microns, were completely spherical in shape, and were surrounded by a two-layered calyptra. Evidence of mitotic activity in the prothallial cells which surround the embryo and serve as the calyptra is noticeable even before the first division of the embryo has occurred. As a result of these divisions, the calyptra is able to keep pace with the rapidly dividing and enlarging embryo until the embryo reaches an advanced stage. Although sectioned material shows the embryo to be in intimate contact with the cells of the surrounding calyptra, no evidence of any physical attachment was detected. The ease with which embryos of this age were removed from the surrounding prothallial tissues would preclude any suggestion of a physical union between calyptra and embryo, at least at that time.

Embryos examined two days after octant formation, eleven days after fertilization, were noted to consist of sixteen cells, produced as a result of the division of each of the octants. The four octants of the anterior half of the embryo, two inferior and two superior, divided in such a way that a partition wall was formed lying parallel with and anterior to the basal wall. The four posterior octants divided in a similar manner producing another wall parallel to the basal wall but posterior to it. In all of the embryos examined, the orientation of the divisions formed in each octant was similar, in contrast to reports in which other ferns were utilized (Vladesco, 1935).

The next division to take place in each of the sixteen cells was found to be completed by the thirteenth day following fertilization. At this time the embryos consisted of thirty-two cells and had an average size of 100 microns × 108 microns. These divisions which occur simultaneously in each of the sixteen cells of the embryo are oriented parallel to the transverse wall and perpendicular to the basal and median wall. The appearance of the divisions responsible for forming the sixteen and thirty-two cell stages in young embryos of *Todea* are characteristic for this species in that they are formed simultaneously in each of the derived daughters of the octants. This regularity seen to exist in the formation of the post-octant stages of embryo development is in marked contrast to the post-octant stages of development in embryos of *Gymnogramme sulphurea* (Vladesco, 1935) and in *Phlebodium aureum* (Ward, 1954).

INITIATION AND DEVELOPMENT OF APPENDAGES — Soon after the embryo has attained the thirty-two cell stage, the various regions of the embryo begin to display signs of differentiation.

The first organ to be clearly distinguishable is the foot. Although the proponents of the octant concept of fern embryology (Hofmeister, Pringsheim, Campbell, etc.) believed this organ to be formed exclusively from the posterior-superior octants, recent investigators (Ward, Cross,

Vladesco) have shown for their respective species that the foot is derived from most of the upper or superior portion of the embryo including both anterior and posterior hemispheres. Embryos of *Todea* examined in this study may be characterized as having the entire superior half of the embryo, posterior and anterior regions, relegated to the formation of the foot.

By the fifteenth day after fertilization several divisions have taken place in the foot region. These divisions are for the most part anticlinal and are in position parallel or slightly inclined to the basal wall. These cells, tetrahedral in shape, can be distinguished from the cells of the inferior region by their large size and their lower cytoplasmic content. As the embryo enlarges, the cells of the foot region undergo several periclinal divisions, thereby becoming rather isodiametric. By the seventeenth day following fertilization the foot is clearly discernible and easily recognized by its large, isodiametric, highly vacuolated cells which stand out in sharp contrast to the meristematic-appearing cells of the inferior hemisphere in which anticlinal and periclinal divisions are occurring rapidly (Fig. 5). Once the superior half of the embryo has produced what can be easily identified as the absorbing organ of the embryo, succeeding divisions occur with less rapidity and regularity and the region can be seen to increase in size by cell enlargement rather than by cell division.

A distinct boundary between the foot region and the prothallial cells which form the calyptra is evident in embryos of *Todea barbara* until a relatively advanced stage. An embryo eighteen days old shows the highly vacuolated cells of the foot to be closely appressed to the cells of the calyptra although no protrusion of the foot cells into the prothallial tissue is evident (Fig. 6). As the embryo continues to grow and the globular mass of tissue begins to change shape, the cells of the foot region are seen to increase in size, many becoming several times longer than wide. Protrusion of these cells of the foot between the adjoining cells of the calyptra is not as distinct as it appears to be in the accounts given by Ward (1954) for *Phlebodium* or Cross (1931) and Campbell (1892) for *Osmunda*.

Although the cells of the foot region are closely appressed to the cells of the prothallus, no destruction of prothallial cells, as described by Campbell (1892), was noted to occur in any of the sections examined. That the inter-locking of foot and prothallial cells is not as pronounced in embryos of *Todea* as it may be in other species is indicated by the relative ease with which older embryos, those bearing recognizable but undeveloped organs, may be removed from the surrounding prothallus.

At an advanced stage, the cells of the foot and those of the prothallus are similar in size and shape; nevertheless, the boundary between foot and prothallus can still be readily distinguished. The foot being notably an organ concerned with the nutrition of the embryo accumulates large amounts of reserve food material in the form of starch. These starch-filled cells of the foot region can be clearly distinguished from neighbouring cells of the prothallus which have the same general appearance but do not possess abundant food material.

At all stages in the development of the embryo, the foot region is clearly distinguishable from the other regions of the embryo. The cells of the superior or foot region are large, thin-walled, and contain abundant starch, while those of the inferior region are highly protoplasmic, densely staining cells which are undergoing frequent divisions.

The first recognizable sign of differentiation to appear in the inferior hemisphere of the young embryo is evident in embryos eighteen days after fertilization. By this time the embryo consists of a considerable mass of tissue possessing a well-defined foot which occupies the superior half of the embryo. The inferior half of the embryo, by contrast, is made up of a great number of meristematic cells bearing evidence of increased mitotic activity in this region (Fig. 6). Up to this time divisions occurring in both the anterior and posterior inferior pairs of octants have produced two regions which are clearly discernible. (1) An outer epidermal-like layer, consisting of one or two layers of elongate cells formed by a preponderance of anticlinal divisions in the inferior half, and (2) an inner layer of smaller isodiametric cells
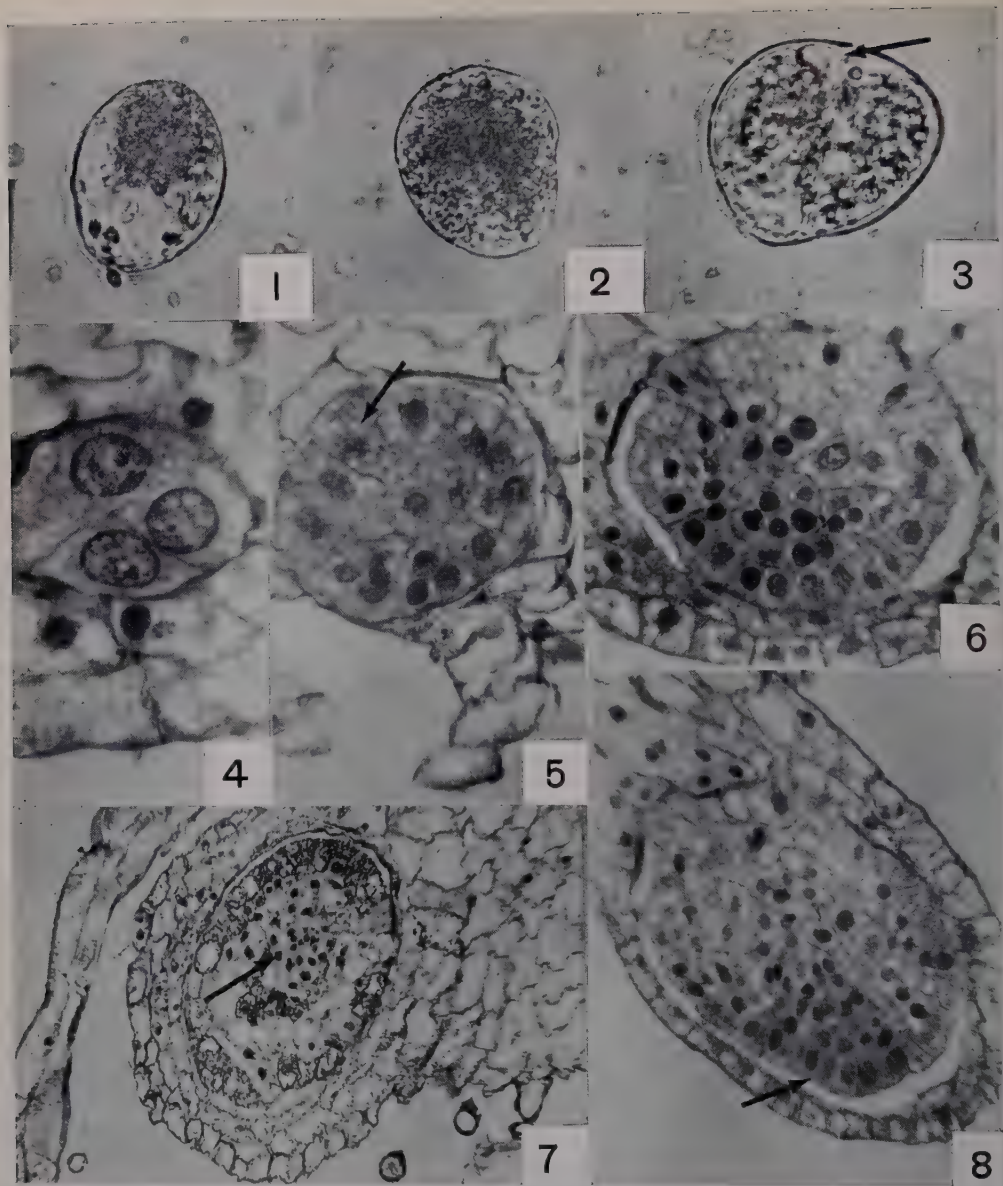
FIGS. 1-8 — Figs. 1-3. Young embryos isolated during the very early stages of development. ×
400. Fig. 1. Embryo, isolated 4 days after fertilization. Fig. 2. Embryo, isolated 5 days after fertili-
zation, showing a more even distribution of cytoplasmic material. Fig. 3. Isolated 6-day-old
embryo showing position of first or basal wall (indicated by arrow). Figs. 4-8. Stages in the
development of the contained, naturally growing embryo. Fig. 4. Quadrant embryo sectioned
7 days after fertilization. Section is parallel to the long axis of the prothallus and at right angles
to the long axis of the archegonium. × 520. Fig. 5. Median longitudinal section of embryo 17
days after fertilization. The foot region is composed of large cells (indicated by arrow). The
section is parallel to the axis of the archegonium and to the long axis of the thallus. × 280.
Fig. 6. Embryo sectioned 18 days after fertilization displaying an outer region of elongate cells,
and an inner region of isodiametric cells in the inferior hemisphere. Fig. 7. Procambial elements
in the central area of the embryo. The section is in the long axis of the archegonium and at right
angles to the long axis of the thallus. × 120. Fig. 8. Section of an embryo 20 days after fertiliza-
tion displaying prominent leaf initials (indicated by arrow). Section as in Fig. 5. × 240.

which occupy the area between the transverse partition wall and the elongate surface cells. It is in this central area of the embryo that the first notable signs of cellular differentiation begin (Fig. 7). Divisions occur rapidly in this area and cellular differentiation proceeds until one is able to recognize a central zone of provascular tissue, reminiscent in some respects of the "nodule" of vascular tissue which forms in tissue cultures of seemingly parenchymatous elements under a variety of experimental conditions (Gautheret, 1957; Steward *et al.*, 1958b; Wetmore & Sorokin, 1955; Reinert, 1959). Only after this area has formed does the differentiation of the various organs; stem, leaf, and root proceed.

The leaf is the first of these organs to form and regularly develops from the cells of the anterior-inferior octant. However, by the time the embryo is eighteen days of age, recognizable leaf initials were present in all embryos examined. Anticlinal divisions occurring in the outer or epidermal layer of cells give rise at this stage to an apical cell which can easily be distinguished as being tetrahedral in shape. The leaf initial then begins to produce successive segments from its lateral surfaces which give rise to a group of leaf initials. From this stage on, the presence of a single apical initial is not always evident in sectioned material since it often appears to have been supplanted by a group of apical cells, primary derivatives of the original cell. This condition has been recorded for *Phlebodium aureum* (Ward, 1954) and more recently for vegetative fronds of *Osmunda cinnamomea* (Briggs & Steeves, 1958).

Soon after the appearance of a succession of leaf initials, active cell division rapidly ensues in the area directly behind the initials. This stage in leaf development is usually characterized at first by a random orientation of divisions in the sub-surface layer which gradually gives way to a more orderly pattern of periclinal divisions as seen in a twenty-day old embryo (Fig. 8).

By continued cell divisions in this central area, the anterior-inferior quadrant begins to bulge distinctly, denoting the first macroscopically visible evidence of leaf initiation. The formation of a protrusion in what up to this point, twenty-two days, had been a seemingly homogeneous, globular mass of tissue, causes the inferior hemisphere of the embryo to increase in size. The surrounding calyptra, at this stage no longer dividing, is, nevertheless, plastic enough to compensate for the increased size without rupturing. For a time the developing leaf grows out at an angle of forty-five degrees to the embryo axis and is parallel to the surface of the prothallus. This phase of growth is short-lived, for in prothalli sectioned twenty-eight days after fertilization the leaf, still surrounded by an intact calyptra, had begun to grow upward and curl over the stem region (Fig. 9). This is suggestive of the early stages in crozier development as recorded by Steeves & Briggs for *Osmunda cinnamomea* (1958).

The cells of the outer or epidermal layer of the embryo begin to elaborate oil and mucilaginous materials. The secretory surface cells completely surround the embryo except at places where organ initials have formed. The outer cells of the embryo are not the only ones to function as secretory cells in embryos of *Todea*. Sections through embryos thirty days old show that the parenchyma cells surrounding the central zone or "nodule" of tissue also become filled with mucilaginous material and at this stage form a boundary within the confines of which the provascular tissue develops (Fig. 13).

Procambial tissue, initially evident in the central area of the embryo twenty-two days after fertilization, develops continuously and acropetally from this region into the growing leaf. By the time the embryo is twenty-five to twenty-eight days of age a continuous strand of procambial tissue is evident extending from the central region to the apical portion of the curled leaf (Fig. 9).

The formation of stem and root follow closely once the leaf has been initiated. Although in embryos of *Todea* the leaf is the first of the vegetative organs to be differentiated, leaf initials being apparent in eighteen-day old embryos, it is not until one or two days later that the stem and root initials can be distinguished. In sectioned embryos twenty to twenty-two
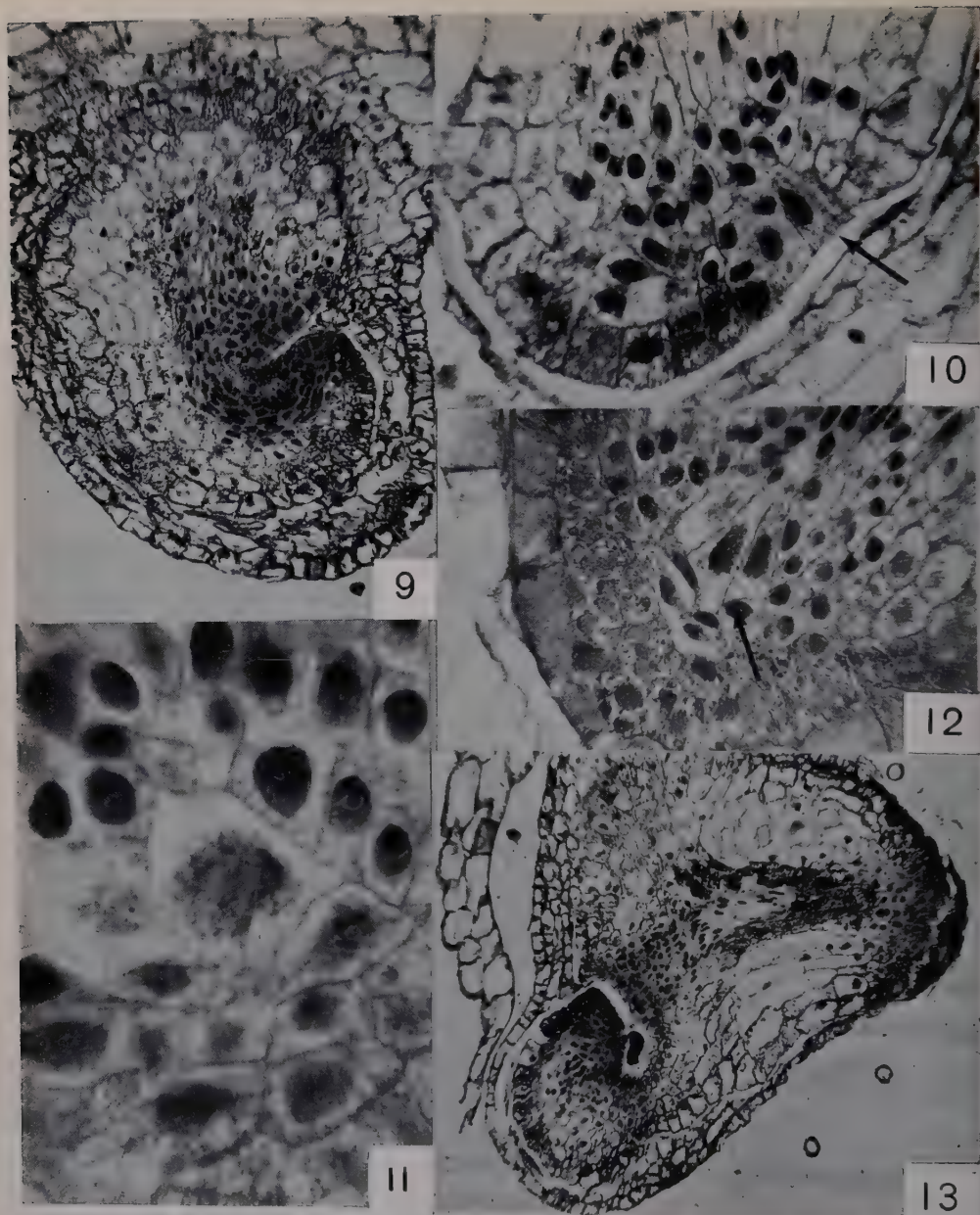
FIGS. 9-13 — Stages in the development of the contained naturally growing embryo. Fig. 9. Section of embryo 28 days after fertilization. The growing leaf has curled over the stem area and procambial tissue can be seen extending from the central region of the embryo into the developing leaf and stem. Sectioned as in Fig. 7. × 101. Fig. 10. Stem initial (indicated by arrow) clearly recognized in sectional embryo 22 days after fertilization. × 284. Fig. 11. Division occurring in the endogenous root apical cell. × 607·5. Fig. 12. Root apical cell (indicated by arrow) which has recently completed a division shows the rhomboid shape characteristic for this species. Well formed root cap region is visible to the left. × 284. Fig. 13. Embryo sectioned 30 days after fertilization showing well developed leaf still enclosed by the calyptra and the root which is just emerging. Provascular tissue can be seen extending from the central region into the developing organs and is surrounded by mucilaginous cells. Section is parallel to tne long axis of the archegonium and oblique to the long axis of the thallus. × 81.

days old, initials of stem and root are quite conspicuous, having been noted to form almost simultaneously in opposite regions of the embryo.

The stem is derived from the surface cells of the anterior portion of the embryo and occupies a position midway between foot and leaf. In its early development, stem formation is characterized by anticlinal divisions of the surface cells. These divisions appear to be formed in continuation of anticlinal divisions which give rise to the leaf initials. The anterior-inferior portion of an eighteen-day old embryo shows anticlinal divisions to be occurring with equal rapidity in both the lower and median portions of the embryo.

As a result of the rapidity with which anticlinal divisions occur in the presumptive stem area, a stem apical cell is produced and is clearly recognizable in embryos sectioned twenty-two days after fertilization (Fig. 10). The apical cell of the stem in *Todea* is approximately four times as long as wide, triangular in shape, with the flat surface oriented to the exterior. The presence of a single apical cell could be detected in embryos twenty days of age. There appears to be no question that a single cell is actually active early in the development of this organ.

As the stem area develops it is noted to encroach more upon the superior half of the embryo, the foot area, than the inferior half, now occupied by the developing leaf. It seems pertinent to note that although the primary divisions which give rise to the apical cell of the stem are formed in a median position of the anterior portion of the embryo the surrounding prismatic cells are seen to occur in both superior and inferior portions. It then appears that the stem is formed in a median position of the anterior part of the embryo and as it grows both hemispheres become involved in its development.

The cells of the embryo directly beneath the stem initials undergo a number of divisions, mainly in a periclinal plane, until the entire stem area appears meristematic. Unlike some of the more advanced ferns at this stage, where a distinct protuberance is noted in the stem region (Ward, 1954; Vladesco, 1935), this area in *Todea* embryos appears level. More-

over, in contrast with these other species, the stem area of *Todea* retains a flattened appearance throughout the development of the embryo.

By the time the embryo is twenty-five days old recognizable procambial strands, first evident in the central region of the embryo, begin to develop towards the stem region. Embryos twenty-eight days of age, which were sectioned to show the developing stem and leaf areas (Fig. 9), indicate that the procambial tissue of the stem is not entirely separate from that directed into the leaf. As the procambium develops in the direction of the stem area it is seen to connect with the strands entering the developing leaf imparting some continuity to the provascular system.

Root development in embryos of *Todea barbara* is evident in the posterior-inferior portion of the embryo twenty to twenty-two days following fertilization. Prior to the time of root initiation divisions taking place in this part of the embryo were found not only to be less frequent than those occurring in the anterior, or leaf portion, but the divisions were initiated in a less orderly fashion, the partition walls having apparently no preferred orientation. As a consequence, the early stages in the development of the posterior-inferior quadrant of the embryo consist of a number of haphazardly arranged cells. In sectioned twenty-day old embryos, one of the cells located inside the outer limiting layers of the embryo appears more prominent than its neighbours. This cell, larger than the surrounding cells, possesses a rather large nucleus and a small amount of cytoplasmic material in comparison with other cells in the same general area. The position occupied by this cell coupled with its histological features appropriately characterize it as the apical cell of the root. At the same time, distinguishable leaf initials are present in the anterior portion and the apical cell of the stem is also apparent.

The apical cell of the root, unlike that of the stem, does not originate in the lower part of the inferior hemisphere but appears to form in the posterior-inferior portion of the embryo at a region close to the transverse and basal walls. In this respect the apical root cell is seen to arise almost diametrically opposite the stem. The root

apical cell at first appears rather rectangular in outline; however, in later stages it assumes a more characteristic shape. Sections cut through the root region of a twenty-five-day old embryo show the apical cell to be still a four-sided figure although it is no longer rectangular but rhomboid in shape (Figs. 11, 12). The apical cell undergoes a series of anticlinal divisions to produce a number of wedge-shaped daughter segments which divide periclinally to yield cells destined to contribute not only to the formation of the root cap but also to the formation of the cortex.

The apical cell of the root is clearly discernible throughout all the latter phases of embryonic development and at no time was any evidence perceivable which would indicate that its function might be given over to a group of initials as occurs in stem and leaf.

Almost from its inception close to the center of the embryo, the root is provided with procambial tissue emanating from the central region. As the root develops, strands of procambium, bordered by secretory parenchyma cells, can be noted to extend from the center region to a point directly behind the root apex. Several days before the enlarged embryo ruptures the calyptra, the procambial strand connects with those of stem and leaf. At this time, any indication of a central region or zone in the embryo concerned with the initial production of procambium is obliterated by the connected and continuous arrangement of developing provascular tissue.

It is not until thirty days after fertilization that the embryo of *Todea barbara* emerges from the calyptra. Sectioned embryos of this age have both a well developed leaf, which has grown upward and curled over the stem, and a well developed root, which is beginning to break through the covering calyptra (Fig. 13). After examining over one hundred fertilized prothallia, it can be stated that in the great majority of cases the root is the first organ of the embryo to rupture the calyptra and only in a few instances was the leaf observed preceding the root through the calyptra. Both organs are ultimately freed by the thirty-second day after fertilization whereupon the leaf begins to straighten and grow out over the side of the prothallus rather than through the apical region as is usually the case for the higher ferns (Wardlaw, 1955; Vladesco, 1935; Bower, 1926; Campbell, 1918). It is a characteristic of the embryos of this species that following their emergence from the calyptra both leaf and root, developing in opposite directions, grow out at right angles to the long axis of the prothallus. *Todea*, however, is not unique in this respect, a similar pattern of growth having been reported for embryos of *Osmunda cinnamomea* (Cross, 1931).

Discussion

The growth of the embryo of *Todea barbara* is characterized by an extended period of containment within the immediately surrounding calyptra tissue. The vegetative organs of the young sporophyte are rather large and well developed even before they emerge from their confined position. These features which have also been observed in developing embryos of *Osmunda cinnamomea* (Cross, 1931; Campbell, 1892) and *Osmunda claytoniana* (Campbell, 1892) have been taken as an indication of the very primitive nature of the Osmundaceae. On the weight of the embryological evidence obtained in this study one is led to agree that *Todea*, like *Osmunda*, represents a primitive class of ferns. Cross (1931) interprets the embryology of *Osmunda cinnamomea* as depicting not only a primitive type of fern but one which has a closer affinity to the eusporangiate than the leptosporangiate level. His arguments in favor of this hypothesis are: (i) the origin of the leaf, stem, and root from the same half of the embryo and the foot from the other half; (ii) the occasional presence of an incipient suspensor.

It is clear from the investigations of Ward (1954) and others (*see* Bower, 1926) that the Osmundaceae are not the only family of ferns having their vegetative organs formed from the same half of the embryo, but that this pattern of organ development is also noted in the Polypodiaceae. In the present study it was early seen that after the egg of *Todea* was

fertilized, the flattened surface, characteristic of unfertilized eggs, enlarged in the direction of the neck canal of the archegonium. In many cases, the distribution of cytoplasmic materials into this newly expanded portion of the young embryo was not coincident with its growth so that a highly vacuolated condition resulted. Cross (1931) regards this vacuolated portion of the young embryo of *Osmunda* as a " rudimentary suspensor " and suggests that the presence of an incipient suspensor is a point in common with the eusporangiate ferns. From the present study it appears that the vacuolation observed in the young embryos is produced as a consequence of fertilization and to attach any morphological significance to it at the present time is without support. Embryological data obtained in this study would thus seem to point out a relationship of the Osmundaceae with other leptosporangiate ferns. Even though considerable variation is evident in the timing of events, the overall picture of embryo development is similar.

The position of the first division of the embryo in *Todea* conforms to the generally accepted pattern for most leptosporangiate ferns; the wall being laid down in the axis of the archegonium at right angles to the long axis of the prothallus. However, considerable variation in this pattern was noted to exist in some embryos which had this first division oriented towards the apex of the prothallus. Vladesco (1935) and Cross (1931) are among those who have reported a similar situation occurring in other species of ferns. In *Todea*, and probably also in *Osmunda*, the position which this first division wall occupies, in some cases being almost parallel to the axis of the thallus, is responsible for the development of leaf and root towards the sides of the thallus rather than towards apex and base. In the majority of leptosporangiate ferns studied, (Ward, 1954; Campbell, 1892; Atkinson, 1894; Shaw, 1898; Bower, 1926), the first division is without exception at right angles to the anterior-posterior axis of the prothallus in a plane of the archegonium. The position of this first division, which separates leaf and stem from root, is such that one can easily perceive the developing leaf

emerging from the calyptra to grow forward and upward through the apical notch of the prothallus. Consider now the situation as it occurs in *Todea*. The first wall is frequently oriented at an angle to the anterior-posterior axis of the thallus. In this position, the leaf will emerge from the calyptra and grow not toward the prothallial apex but toward the margin of the prothallus. Whether or not we recognize Bower's interpretation of the embryo as a " primitive spindle " (1922), we must agree from the foregoing observation that the polarity of the young embryo has been determined at the time the first wall is formed. As yet, no information is available which would enable us to determine whether the first wall is necessary before the polarity of the embryo is definitely established or whether the factors which govern polarity are inherent in the undivided embryo.

Earlier workers in fern embryology (Goebel, 1887; Campbell, 1918; Hanstein, 1865; Conard, 1908) held the position of the quadrant and octant walls to be of special morphological significance in the further development of the young embryo. The results obtained by these workers indicated the transverse wall to form prior to the median wall. Thus the classical accounts of the early segmentation of leptosporangiate fern embryos maintained that the transverse wall was actually the quadrant wall and the median wall the octant. Vladesco (1935) has shown that a great many higher ferns do not rigidly follow the classical pattern of segmentation but have the median wall formed before the transverse wall. Cross (1931) finds the second division in *Osmunda* to be either median or transverse. In the present inquiry, the second partition wall to form in embryos of *Todea* is, without exception, the median wall; and the transverse wall forms only after the previous division has been completed.

That the orientation of these primary divisions is not influenced by external factors has been indicated by Vladesco (1935) who found that the position of the first three walls of the zygote remained constant even though the prothalli were grown under varying conditions of culture, illumination, and gravity.

In preliminary experiments, fertilized prothalli of *Todea* were placed in sterile culture on nutrient media containing varying amounts of sucrose and the embryos allowed to develop. Results of this study have indicated that the early segmentation pattern of the young embryo is not visibly affected by the concentration of sugar in the nutrient medium. In short, it would appear that the factors responsible for the early division of the young fern embryo are inherent in the embryo itself or are produced as a result of the zygote-prothallial relationship. Some information is available which suggests that the enclosed growth habit of the young embryo, situated within the turgid prothallial tissue and surrounded by the turgid cells of the calyptra, might actually affect the orientation of cell divisions and the early organization of the developing embryo (Ward & Wetmore, 1954). Indeed, in considering the causes responsible for cell orientation one must take into account not only the chemical but also the physical factors which may possibly be involved.

The foot is the first structure evident in young embryos of *Todea barbara* and, as in other ferns, serves as an absorbing organ for the growing embryo. Histological examination of the foot region has shown that the cells of this region do not penetrate into the surrounding prothallial tissue until very late in development.

Throughout most of the growing stages, the cells of the foot region, although in contact with the prothallial tissue, do not show the characteristic interlocking generally associated with this organ. If the embryo is attached at all to the parent prothallus, this attachment must be a very feeble one since embryos in rather advanced stages of development are easily removed from their position in the prothallus without visible damage. It may seem surprising that the embryo, originally derived from one of the cells of the axial row, early in its development, becomes separated from the surrounding cells and, from all observations made in this study, remains essentially free until an advanced stage. This condition is, however, not without its parallel, for in studies carried out on the growth of various tissues in

sterile culture (Muir *et al.*, 1958; Gautheret 1955; Steward *et al.*, 1958a; Torrey *et al.*, 1957) it has been found that alteration of the type of culture media will produce extremely friable clusters of tissue from which single cells can be obtained. As Torrey *et al.* (1957) have pointed out, the friable condition is probably due to a breakdown of the cementing pectic material of the intercellular layer through various enzymatic activities. That some similar situation is responsible for the release of the fern embryo at an early stage is suggested by the seeming similarity which exists in these two diverse systems. However, it has not been possible to check this situation as yet.

Very early in the development of the embryo, before leaf, root, and stem are apparent, the central area of the embryo delimited by rectangular cells of the surface layers shows evidence of cellular differentiation. As the various organs of the embryo are initiated and develop, differentiation in the center region advances until one clearly recognizes a central aggregation of procambial elements. It is in this region that procambium is initiated and from this region that it develops acropetally into the primordial organs of the embryo. Similarities which exist between the development of isolated cells from the secondary phloem region of carrot roots (Steward *et al.*, 1958a, 1958b, 1958c) and the development of a fern embryo appear to be dependent on the attainment of an adequate multicellular condition. Once a three-dimensional mass of tissue is evident, internal differentiation is seen to occur after which, and only after which, does organ formation ensue. This condition is well documented for the growth of single cells or cell aggregates (Steward *et al.*, 1958b) and it is felt that the observations recorded in this study will serve to establish this growth pattern as a more general type.

As is usually the case in the development of the fern embryo, the leaf is the first vegetative organ to be produced. Unlike most of the other leptosporangiate ferns, the developing leaf of *Todea* does not grow directly out of the calyptra as it does in *Polypodium* (Ward, 1954) and *Gymnogramme* (Vladesco, 1935). Instead of

growing out at an angle of forty-five degrees and rupturing the calyptra, the developing leaf is seen to begin a period of upward growth during which time it curls over the stem apex.   This circinate pattern of leaf growth has been noticed for *Osmunda* (Campbell, 1892; Briggs & Steeves, 1958), although Cross (1931) makes no mention of its occurrence in his study.

In the development of the various organs of the embryo it has been found that each has its beginning from a single apical cell. These apical cells have been observed, in embryos of *Todea barbara*, to arise rather late in development, no apical cell being noted before the embryo has attained a considerable mass.

In following the initiation of the apical cells of the various organs and the type of derivative cells formed, it was apparent that while both the leaf and stem apical cell seemed to be replaced early in their ontogeny by a group of apical initials, the root maintains a distinct apical cell throughout its entire embryological development.

Although in embryos of *Phlebodium aureum*, Ward (1954) found no evidence for the presence of a single apical cell in the development of the root, single root apical cells have been noted for *Adiantum concinnum* (Atkinson, 1894) as well as for *Dennstaedtia punctilobula* (Conard, 1908). In *Gymnogrammae sulphurea*, Vladesco (1935) pictures a single apical cell as being persistent in the embryonic development of the root.   A persistent root apical cell has also been observed in developing embryos of *Osmunda* by Cross (1931) and Campbell (1892).   Bower (1889) considers that no strict uniformity exists in the meristems of the root in *Osmunda* and *Todea*.   At times he observed a single apical cell in the root region of *Osmunda regalis* but in no instance was a single apical cell found in sections of root apices of *Todea barbara* although he takes no issue with the findings of vanTieghem and Douliot who, he indicates, record the presence of a single apical initial in the roots of *Todea barbara*.

The present work suggests that root development in embryos of *Todea* is first evident in the delimiting of an endogenous cell in the posterior-inferior portion of the embryo which assumes a characteristic rhomboid shape and thereafter functions as an apical cell in the further development of the root.

The early stages of leaf and stem development recorded in this investigation appear to confirm in most respects to findings reported for other leptosporangiate ferns.   However, one variation in the sequence of organ formation is worthy of further comment.   The initiation of the various vegetative organs in embryos of *Todea* has been observed to follow a regular pattern.   The leaf is the first organ to appear, followed, at an interval of two to four days, by the formation of both stem and root apparently arising almost simultaneously in almost diametrically opposite regions of the embryo.   That this sequence of organ initiation is not followed by other species is evident from the work of Ward who notes that in *Phlebodium aureum* the stem is the last of the vegetative organs to form, being visible only after leaf and root have been initiated.   What morphological significance should be attached to the variations observed in the sequence of organ initiation and formation in the various groups of leptosporangiate ferns is not known at the present time.   From this study it appears evident that while developing embryos of *Todea barbara* may show considerable variation when compared with other related groups, the totality of their morphological expression is considered to be representative of the leptosporangiate type.

Summary

The present study records the various stages through which the embryo of *Todea barbara* passes as it attains maturity.   An examination of these stages, characteristic for various ferns and groups of ferns, has demonstrated that *Todea barbara* is more closely related to the leptosporangiate than the eusporangiate ferns.   Although the timing and sequence of the early divisions and the initiation of organs differed somewhat from accounts given for other leptosporangiate ferns, the general scheme of development was similar.

An attempt has also been made to clarify the terminology frequently used to describe the development of the young fern embryo. Recent advances in embryological concepts has necessitated a redefinition of certain useful terms in order that they may adequately serve the present-day workers in this field.

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THE MORPHOLOGICAL DEVELOPMENT OF THE FRUIT OF *PISUM SATIVUM*, VAR. *ALASKA**

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Introduction

A few years ago (Linck, 1955) it was observed that in the pods of the *Alaska* variety of *Pisum*, all the 5-7 ovules do not mature into seeds. The cause for this phenomenon is not known. This present study was made to obtain morphological data on the growth of the pod and ovules of the Alaska variety of *Pisum* in order to understand the causes of embryo failure.

Materials and Methods

Seed of the *Alaska* variety of *Pisum sativum* L. obtained from the Green Giant Company, Le Sueur, Minnesota, was used in this study. The seeds were soaked for 2 hours in distilled water and then placed between layers of filter paper in Petri dishes. Germination was allowed to proceed at room temperature for 48 hours. At the end of this time the germinating seeds were selected for uniformity and planted in 4-inch pots in a mixture of soil, sand, and peat moss (in the ratio of 3:2:1). Unless otherwise stated the plants developed to maturity in a controlled environment room which had a 12-hour light period at 75°F followed by a 12-hour dark period at 60°F. Maximum light intensity at the stem apices of the plants was about 1500 f.c. from incandescent and fluorescent lights. Morphological and histological data were obtained on pod development on plants at different stages of maturity.

Throughout this paper the intact ovary (or ovulary) regardless of stage of development will be referred to as the pod. When the ovules or seed are removed from
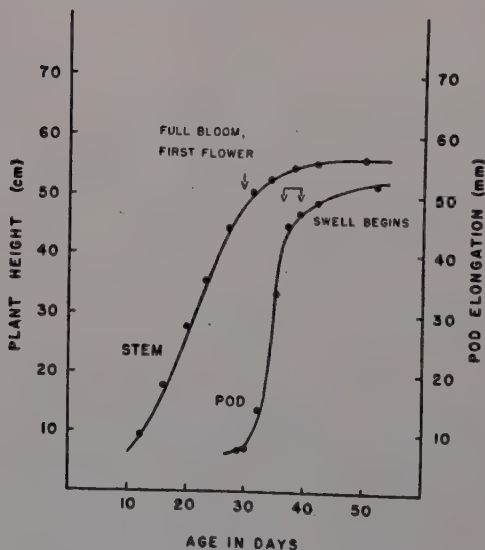
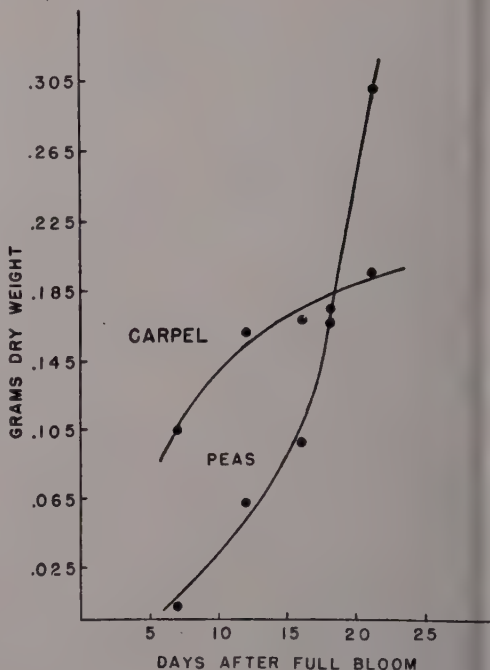the pod, the structure remaining is the carpel and will be referred to as such.

Results and Discussion

Under the environmental conditions of the controlled environment room, anthesis at the first bloom node occurred on the 27th to 30th day after planting.   The peas in all pods were considered to be mature at about 50 days.   Following anthesis the pod elongates and then increases in size at right angles to the long diameter — a phenomenon referred to as pod " swell ". Plant height and pod length measurements were made at intervals and are plotted in terms of number of days from planting of the seed (Graph 1).   The time of anthesis of the first flower and the onset of pod " swell " are indicated.   Elongation of the stem as indicated by the plant height was found to approach a maximum rate a few days after anthesis of the first bloom and the subsequent elongation of the pod in this flower.   The increase in the short diameter of the first pod begins on about the 35th day.

Measurements were made at intervals of the growth of the primary root of plants growing in a complete mineral solution

GRAPH 2 — Ontogenic changes in the dry weight of the carpel and of the peas.   Averages of 10 plants per harvest.

GRAPH 1 — The rate of growth in height of pea plants and the elongation of the first bloom-node pod as a function of the age of the plants.

in the controlled environment room.   The rate of primary root elongation increased through the 18th day after germination but a diminished rate was observed on the 23rd and 29th day.   Thus the rate of root growth is reduced several days before the onset of flowering.

In order to determine the relative rates of growth of the parts of the pod, i.e. the carpel and the peas, dry weight data on both of these morphological parts were obtained from the 7th day after anthesis to the 21st day.   The results (Graph 2) indicate that the dry weight of the ovules increased approximately 50-fold during this period.   Maximum rate of increase in dry weight of the carpel was reached at about 12 days after full bloom, although some increase was made up to the final harvest.   These results suggest that there may be a rapid export of translocatable materials from the carpel to the ovules, especially during the last 10 days of the development of the ovules.

In order to obtain an estimate of the number of ovules which never reach maturity as seeds, mature pods from the first bloom node were opened and the ovules classified as to the stage of development. Ovules approximately 1 mm or less in longest diameter were called " vestigial ", ovules longer than 1 mm but not of full size were classed as "small " and those ovules which had reached maximum development and would have been processed for canning in a commercial operation were classed as " normal ". Table 1 gives the results of data based on the number and class of the ovules from pods at the first bloom node which had developed in the greenhouse or controlled environment room at Columbus, Ohio, or in the field at Le Sueur, Minnesota. Approximately one-fourth to one-third of the ovules were found to be " vestigial " in size, one-fourth to one-third " small " and the remainder (one-third to one-half) were found to be " normal ". From observations of the mature pod it was apparent that the distribution of these 3 classes of ovules was not at random in the pod. Table 2 gives the results of a study in which the class of each ovule at each position within the pod at the first bloom node was noted. Position one in the pod was the ovule nearest the peduncle. The vestigial ovules were found most frequently at the " end " positions

TABLE 2 — THE PERCENTAGE DISTRIBUTION OF THE 3 CLASSES OF OVULES IN MATURE PODS

Class	Ovule position in pod*						
	1	2	3	4	5	6	7
Vestigial	59	22	0	0	19	41	54
Small	41	59	41	12	31	44	46
Normal	0	19	59	88	50	16	0

*Values given by ovule position in the pod beginning, at the peduncle end. Data from averages of 32 first bloom node pods, from plants grown in the controlled-environment room.

of the pod and the " normal " ovules at the " center " positions. Ovules classed as " small " were found less frequently in the " center " positions. By the time the pod is mature the vestigial ovules are dry and only a remnant remains. It seemed likely that either fertilization had not occurred or that the growth of the embryos in these ovules had been arrested in their early development. Therefore an histological study was made at different stages of pod development.

Pods were removed from plants at 5 stages: (a) 24 hours prior to anthesis, (b) on the day of anthesis, (c) 48 hours after anthesis, (d) 96 hours after anthesis, and (e) 7 days (168 hours) after anthesis. Examination of the flowers 24 hours prior to anthesis indicated that the anthers usually had not dehisced at this time. Pollen appears to be shed during the day of anthesis, which for all of these experiments was defined as the day on which the " standard " and " wing " petals reflexed back from their closed position. Flowers or pods were removed from the plants and immediately killed and preserved in F.P.A. (5 per cent formalin, 5 per cent propionic acid, 90 cc. of 50 per cent ethyl alcohol). The preserved material was carried through the usual paraffin embedding procedure and sectioned with a rotary microtome. The sections were stained with 50 per cent alcoholic safranin-0 and 95 per cent alcoholic fast green. Serial longitudinal sections, 12 microns thick, were made at right angles to the pod sutures. Forty-eight hours after full

TABLE 1 — THE DISTRIBUTION OF 3 CLASSES OF OVULES IN THE POD AT MATURITY

(*Data obtained from field, greenhouse and controlled-environment room locations*)

Location where sample was obtained	% of ovules by class*		
	Vestigial	Small	Normal
Le Sueur, Minnesota (field)	35	31	34
Columbus, Ohio (greenhouse)	32	24	44
Columbus, Ohio (controlled-environment room)	25	22	53

*Data were obtained from the first bloom node pod only.

FIGS. 1-7 — Photomicrographs of sections from the ovules of 1 pod of *Pisum sativum*, var. *Alaska*, harvested at 7 days after anthesis. Near-median sections of the ovules and embryos are shown in 1, 2, 4, 6 and 7 with the embryos of 2 ovules (1 and 4) shown in 3 and 5.

bloom the embryo sac contained an embryo at about the 4-cell stage. By the fourth harvest (i.e. 96 hours after anthesis) the embryos have many cells and are spherical. Thus fertilization in the *Alaska* variety, under the experimental conditions used here, probably occurs on the day of anthesis or during the following day. It is possible that embryos could develop

parthenogenetically but this has not been reported for *Pisum sativum* (Wellensiek, 1925). Cooper (1938) reported that fertilization in *Pisum* varieties of *Little Marvel* and *Asgrow's Pride* occurs in the late bud stage, 12-24 hours before anthesis. He reported that the embryos in the varieties which he studied developed from normal fertilization. In the present study

ovules from the 7-day harvest contained well-developed embryos in which the cotyledons were just beginning to differentiate. In the 2 varieties studied by Cooper (1938) cotyledonary differentiation occurred at 6 days following anthesis. Although ovules and their embryos at 96 hours after anthesis appear to be approximately the same size, at 7 days there are striking differences among the ovules of the same pod. Some ovules are about four times as large as others and even greater size differences exist among the respective embryos. This is illustrated in Figs. 1-7. In this series of photomicrographs of 1 representative pod, ovule sections which were near median were selected and photographed at the same magnification so that size comparisons could be made directly. In those ovules where the embryo is small, the ovule is of a smaller size (Figs. 1, 2). The embryos of such ovules (Fig. 3) stain more deeply and the spaces between cells suggest that some kind of anatomical deterioration has occurred. Examination of the vascular connections of these ovules with the pod revealed no differences from those ovules which appeared to be developing normally. Serial sections of more than 30 ovules were studied and in only one was it impossible to find an embryo. It seems likely that failure of fertilization is not a factor in the subnormal development of the ovules arrested early in development.

Normal seed development in pods of *Pisum* has been shown to be accompanied by both a failure or abortion of some ovules and by the lack of complete maturation of other ovules within the same pod. Anatomical study showed that an embryo was developing in each ovule so that lack of fertilization was apparently not a cause for ovule failure. It was found that the embryos in the end positions of the pod become malformed (classed in this study as vestigial) more frequently than do those in center positions. Since all ovules develop apparently normal embryos at least through the 4th day after anthesis, the failure of the ovules at the peduncle cannot be explained by the distance the pollen tube must grow. The fact that ovules at the stylar end also fail frequently also makes this hypothesis

untenable. For *Medicago*, Cooper (1935) found that embryos did not develop in the peduncle end of the pods due to lack of fertilization.

In the present study it was found that the embryo failure began some time between the fourth and seventh day following anthesis. Cessation of ovule growth accompanied that of the embryo and by pod maturity this ovule was dry and withered. A considerable amount of work has been done on embryo abortion in various non-compatible crosses in *Datura*. Sachet (1948) reported that in 48 incompatible crosses in this genus, the endosperm and hybrid embryos had begun development, following fertilization, before some post-fertilization factor brought about abortion. This abortion was apparently connected with the formation of a " tumoral tissue " in the embryo sac. Satina *et al.* (1950) have further investigated these " ovular " tumors and have found that this aberrant cell division continues until the endosperm and embryo are absorbed. No such tumor-like growth has been found in the ovules of *Pisum sativum*, variety *Alaska*, in the present study.

Summary

The morphological development of the flower and fruit of *Pisum sativum*, variety *Alaska* in relation to the vegetative growth of the plant has been studied.

The failure of about one-third of the embryos of this variety of *Pisum* to develop under several environmental conditions was found. This embryo failure was most frequent in the end positions of the ovules in the pod. Ovules with their embryos which developed to normal (canning size) were found to occupy the center positions in the pod most frequently. Under the conditions of growth reported here from about 30 to 50 per cent of the ovules in a pod (at the first bloom node) develop to maturity.

The tentative conclusion is reached from the anatomical study that lack of fertilization is not a cause of embryo failure in the *Alaska* variety of *Pisum*.

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CHEMOTROPISM OF POLLEN TUBES *IN VITRO* IN SPECIES OF *CLIVIA* AND *HAWORTHIA**

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The chemotropic growth of pollen tubes toward tissue or organs of the same species *in vitro* has been reported by workers since the paper of Molisch in 1893. Miki (1954, 1955) has reported the chemotropism of pollen tubes of two species of *Lilium* and one species of *Camellia*. Brink (1924) has reviewed the literature up to 1924 and Tsao's report included a literature survey up to 1949, which indicated that there are only 10 species of plants known for which the chemotropism of pollen tubes has been demonstrated. Recently four additional species of plants were reported to have pollen for which this phenomenon exists (Linck & Blaydes, 1960). It was found that the pollen tubes of *Aloe confusa, Clivia nobilis, Gasteria verrucosa*, and *Haworthia planifolia* would grow toward small segments of floral organs and in addition the pollen tubes of *Clivia nobilis* grew toward segments of the leaves. The present study is an extension of this work and includes some additional species of the genera *Clivia* and *Haworthia*.

Materials and Methods

Pollen was collected on the day of anthesis or the following day from plants grown in the greenhouse. Small segments (usually less than 2×2 mm) of floral parts such as the petal, sepal, anther filament, stigma, style, ovary wall, and ovule were cut and embedded just at the surface of an autoclaved semi-solid medium (1 per cent agar, 10 per cent sucrose, 100 ppm yeast extract) in Petri dishes. Pollen from the same species was placed around the plant parts at a distance of from 1 to 2 mm, using a dissecting microscope. Pollen was placed on the medium either immediately after the tissue segments were embedded or about 1 hour later.

In any one experiment the observations of the growth of the pollen tubes toward

a given tissue were based on 4 to 5 segments (duplicates) and each experiment was replicated at least once. Chemotropism was considered to be demonstrated if 90 per cent or more of the pollen tubes within the first 1 mm from the embedded tissue grew toward the tissue.

Results and Discussion

The pollen tubes of *Clivia miniata* Regel, *Haworthia margaritifera* Haw., and *Haworthia cuspidata* Haw. grew toward various tissues of the flowers of the same species respectively (Table 1). It is interesting to note that pollen tubes of the 3 species grew toward tissue segments from petals, sepals, and anther filaments (*Clivia* only) in addition to segments of the ovary. Thus the chemotropic factor is present in organs not involved directly in the process of fertilization. As it had been found that

the pollen of *Clivia nobilis* (Linck & Blaydes, 1960) would grow toward segments of the leaves of the same species, the pollen of *Clivia miniata* was studied in a similar way. It was found that the tubes grew chemotropically toward leaf segments of *Clivia miniata*.

The results of this study indicate that chemotropism may be common within certain genera such as *Haworthia*, since the phenomenon has now been shown for 3 species. For *Clivia* with 3 known species (Bailey, 1949), pollen tube chemotropism has been found in *C. nobilis* and *C. miniata*.

The compound causing the chemotropic growth of pollen tubes remains to be isolated. Both species of *Clivia* present excellent material for such a study because these species have large pollen grains (advantageous for assay purposes) and because the compound appears to be ubiquitous in the above-ground parts of these plants.

TABLE 1 — SPECIES IN WHICH POLLEN TUBE CHEMOTROPISM WAS FOUND

PLANT	TISSUES TOWARD WHICH POLLEN TUBES OF THE SAME SPECIES GREW
Clivia miniata	Ovule, ovary wall, stigma, style, anther filament, sepal, petal, leaf
*Haworthia margaritifera**	Ovary, stigma, style, sepal, petal
Haworthia cuspidata[1]*	Ovary, sepal, petal

*The ovules were not excised from the ovary because of their small size; the chemotropic factor is presumed to be in both the ovary wall and in the ovules of these species.

Summary

The chemotropic growth of the pollen tubes *in vitro* of 3 species of plants, *Clivia miniata*, *Haworthia cuspidata*, and *Haworthia margaritifera*, toward excised parts of the flowers of the 3 species, respectively, was demonstrated. Pollen tubes of *Clivia miniata* also grew toward leaf segments of the same species.

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THE LIFE HISTORY OF *PODOLEPIS JACEOIDES* (SIMS) VOSS — I. MICROSPOROGENESIS AND MALE GAMETOGENESIS

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Introduction

Podolepis Labill. is an Australian genus of eighteen species, belonging to the family Compositae, tribe Gnaphalioidae. All the species are confined to the mainland, except *P. jaceoides* (Sims) Voss. which is also widely spread in Tasmania and the islands of Bass Strait.

In a previous paper (Davis, 1956) it was suggested, on morphological grounds, that *P. jaceoides* is the most primitive species and the present investigation is an attempt to determine to what extent this conclusion is supported by evidence from other fields.

Comparative studies have been carried out on *P. canescens* A. Cunn. ex DC., *P. longipedata* A. Cunn. ex DC., *P. arachnoidea* (Hook.) Druce, and *P. neglecta* G. L. Davis, all but one of which are thought by the present author to have arisen from *P. jaceoides* by geographic subspeciation.

Material

P. jaceoides is relatively common throughout Tasmania, Eastern Australia, and the eastern half of South Australia. It is a perennial herb from whose root stock usually only a single leafy scape-like stem arises each spring and attains a height of 7-90 cm and terminates in a large golden-yellow capitulum which measures up to 7 cm across the expanded ray florets.

The material on which this paper is based was collected by the author from the grounds of the University of New England in October 1958 and November 1959.

The material of *P. canescens* was obtained from near Broken Hill (western New South Wales) by Mr J. Charley in August, 1958. This is the most widely distributed species of the genus, extending from the western districts of New South Wales and Victoria, throughout Northern Territory and South Australia to Western Australia.

The remaining three species examined, *P. longipedata*, *P. arachnoidea* and *P. neglecta*, were collected by the author at Noosa Heads, 100 miles north of Brisbane, in August, 1958. These species have a relatively restricted distribution in that they occur only in Queensland and Northern New South Wales. *P. arachnoidea* is particularly interesting since it is quite distinct in external morphology and cannot be connected satisfactorily with any other species.

Methods

Material was fixed in F.A.A. and paraffin. Sections were stained with Johansen's safranin heated to 50°C and fast green. Supplementary examinations were made from dissections and aceto-orcein squashes of the fresh material.

The Microsporangium

THE DEVELOPMENT OF THE ANTHER WALL AND MICROSPORE MOTHER CELLS — The pre-archesporial anther is oval in cross-section and shows no tissue differentiation (Fig. 3). Four vertical uniseriate rows of hypodermal archesporial cells then become distinct, and as these expand

radially the anther becomes rectangular. The archesporial nuclei are larger than those of the remaining cells and their cytoplasm stains rather more densly (Fig. 4).

The archesporium is overlaid only by the epidermis, whose cells, at this stage, differ little from those internal to them in the anther. At first the epidermis keeps pace with the developing internal tissues by anticlinal divisions, but soon the fact that it is under pressure is shown by its cells becoming radially flattened and stretched both tangentially and longitudinally. Vacuolation sets in, the nuclei lose their sharp outline, and in the mature anther all that remains of the epidermis is a very thin layer of dead, collapsed cells with ill-defined nuclear remains (Figs. 20-22).

At no stage is the epidermis of adjacent anthers fused, in the sense of an actual tissue connection. The five anther primordia are distinct entities and remain unconnected organically throughout their life. Their so-called " fusion " is brought about by a sheet of cuticle-like thickening being laid down on the outer tangential walls of the epidermis and adhering to that of adjacent anthers to form the anther tube. This thickening first becomes apparent at the microspore mother cell stage and later loses its connection with the epidermis except in the area adjacent to the connective (Figs. 21, 22).

Each archesporial cell divides periclinally to form two daughter cells of equal size — an outer primary parietal cell and an inner primary sporogenous cell — and this localized mitotic activity initiates the four anther lobes (Figs. 5-7). At this early stage each lobe in a cross-section shows a group of six cells which, in their large nuclei and dense cytoplasm, resemble archesporial tissue (Fig. 7). However, only the central one represents the primary sporogenous cell, while the five surrounding ones consist of the primary parietal cell and four others from the adjoining ground tissue. It is from these that the wall layers internal to the epidermis develop.

Increase in size of the primary sporogenous cell and anticlinal divisions of the parietal ones lead to a considerable size difference in these two types of cells, and the anther becomes more pronouncely lobed. The anther wall now consists of two cell layers, the epidermis and the parietal layer (Figs. 8, 9), and it is at this stage that the sporogenous cells undergo their first mitotic division, which is a transverse one (Fig. 8). This is followed by a second mitotic division, in which the spindle is orientated obliquely to the long axis of the anther, with the result that the walls between the daughter cells are also oblique (Fig. 10). The products of this second division of the sporocytes are the microspore mother cells, which are wedge-shaped and biseriately arranged (Figs. 10-12, 15). Concurrently with the second sporocyte division, the parietal layer divides periclinally to give rise to the tapetum and a central layer which divides again periclinally to form the endothecium and the middle layer (Figs. 10-13). The anther wall now consists of four cell layers and no additional cell layers are formed (Figs. 12-15). Its development is shown diagrammatically in Fig. 1.
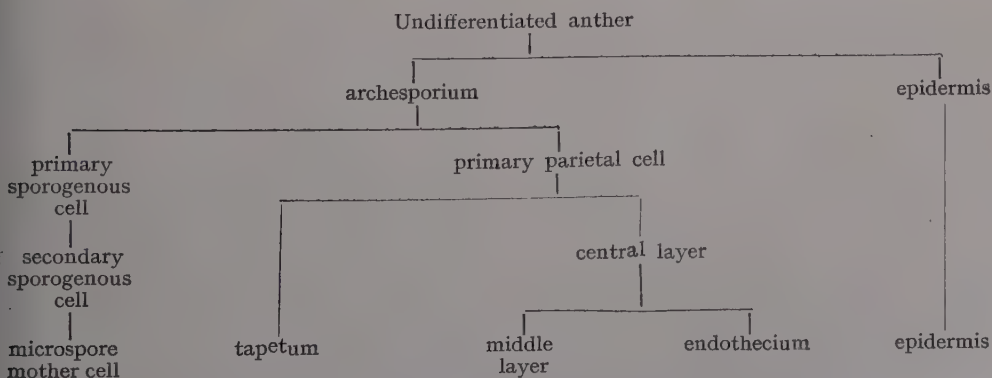

FIG. 1 — The development of the anther wall.

Gradual changes in the shape and dimensions of the cells can be traced in the course of wall formation, and are the the result of pressure exerted by the developing microspore mother cells. At the four-layered stage of the wall, the individual cells are tangentially and longitudinally flattened. Vacuolation of the cytoplasm occurs first in the epidermis but is soon followed by that of the endothecial cells in which a crystal of calcium oxalate becomes a conspicuous feature and persists throughout the life of the cell. The appearance of vacuoles marks the end of cell division and further increase in size of the anther occurs by cell expansion. A further manifestation of pressure on the anther wall is the collapse of the middle layer whose cells lose their cytoplasm and can only be identified by their persisting and distorted nuclei. No trace of the middle layer remains when the pollen grains are binucleate (Fig. 20).

TAPETUM — The cytoplasm of the tapetal cells gradually increases in density, and at the microspore mother cell stage it stains almost as deeply as the nuclei. At no stage, however, are vacuoles present. The tapetal cells then lose contact with each other and, with their large round nuclei (Fig. 27), are very similar to the adjacent microspore mother cells. The tapetal nuclei undergo a series of mitotic divisions (Fig. 2) in the first of which the spindle is always parallel to the long axis of both the cell and the pollen sac (Fig. 28).

The two daughter nuclei then enter a second mitosis simultaneously and two possibilities exist for further development: (1) The two spindles are formed at right angles to the long axis of the cell and coalesce. The chromosomes of both nuclei are arranged in a single metaphase plate which occupies a median position along the long diameter of the cell (Fig. 30). At anaphase one or more chromosomes either lag behind or fail to separate and give rise to bridges when the nuclear membrane reforms (Fig. 31). The number and breadth of such bridges depend on the number and positions of the chromosomes concerned, and result in the formation of a deeply-lobed $8n$ nucleus. These bridges can be seen satisfactorily only in squashes and at oil immersion magnification, (2) The two

spindles are parallel to the long axis of the cell as in the first mitosis (Fig. 32), and the four daughter nuclei are arranged in a linear manner (Fig. 33). These then undergo another division in which their spindles are at right angles to the previous one and, following spindle coalescence, a common metaphase plate is formed (Fig. 34). As in (1), anaphase bridges result in the formation of a deeply- and variously-lobed nucleus which, in this case, is $16n$ (Figs. 35, 36). In one instance only half the chromosomes at the metaphase plate underwent anaphase and this is thought to account for the formation of the 3-lobed and Y-shaped nuclei which occasionally occur (Figs. 37, 38).

In *P. canescens, P. neglecta, P. longipedata* and *P. arachnoidea*, a third possibility also exists: Two of the four nuclei may undergo another transverse division to give rise to a linear series of six nuclei (Figs. 39, 40). Simultaneous longitudinal mitosis then follows in which the six spindles coalesce and the metaphase plate is a very elongate structure. Anaphase bridges are formed as previously, and result in the formation of a lobed, $24n$ nucleus.

Whether the lagging chromosomes or daughter chromosomes, which fail to separate completely, are responsible for the anaphase bridges has not yet been determined, but their presence only at the final mitosis must be associated, in some way, with the orientation and coalescence of the spindles.

A close physiological connection appears to exist between the tapetum and the microspore mother cells and it seems that the influence of whatever stimulus initiates meiosis extends beyond the sporogenous tissue and causes the adjacent tapetal nuclei to react in a "sympathetic" manner by undergoing mitosis. The first mitosis always coincides with the initiation of meiotic prophase in the microspore mother cells and no further divisions of the tapetal nuclei occur after the completion of this stage. This suggests that the time available determines the number of divisions, and perhaps in *P. jaceoides* the duration of meiotic prophase is shorter than in the other species examined, and only permits formation of four nuclei before the final longitudinal division.
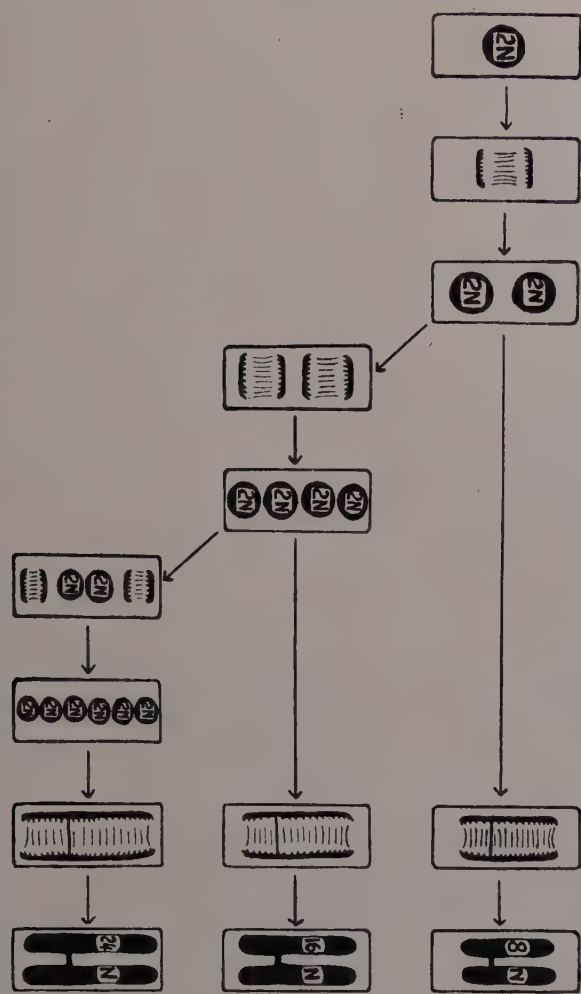
FIG. 2 — Diagrammatic representation of the behaviour of the tapetal nuclei.

Although poor cytological fixation pre-vented a detailed study of meiotic pro-phase there appears to be no exact relation-ship between its stages and the behaviour of the tapetal nuclei, since considerable variation was seen in even adjacent tapetal cells. All, however, undergo the final longitudinal mitosis at about the same time, but the degree of polyploidy in the resulting lobed nuclei depends on the number of previous mitoses and is not necessarily the same in all the cells.

Unfortunately, with the material avail-able, it was not possible to determine the value of N.

Cooper (1933) reported three types of nuclear behaviour in the tapetal cells of angiosperms: (1) the cells remain uni-nucleate throughout their life and no nuclear changes occur; (2) A single mitosis results in 2-nucleate cells; (3) the nucleus may divide twice. Regular divisions lead to the 4-nucleate condition and incom-plete and irregular divisions result in uni- and bi-nucleate cells.
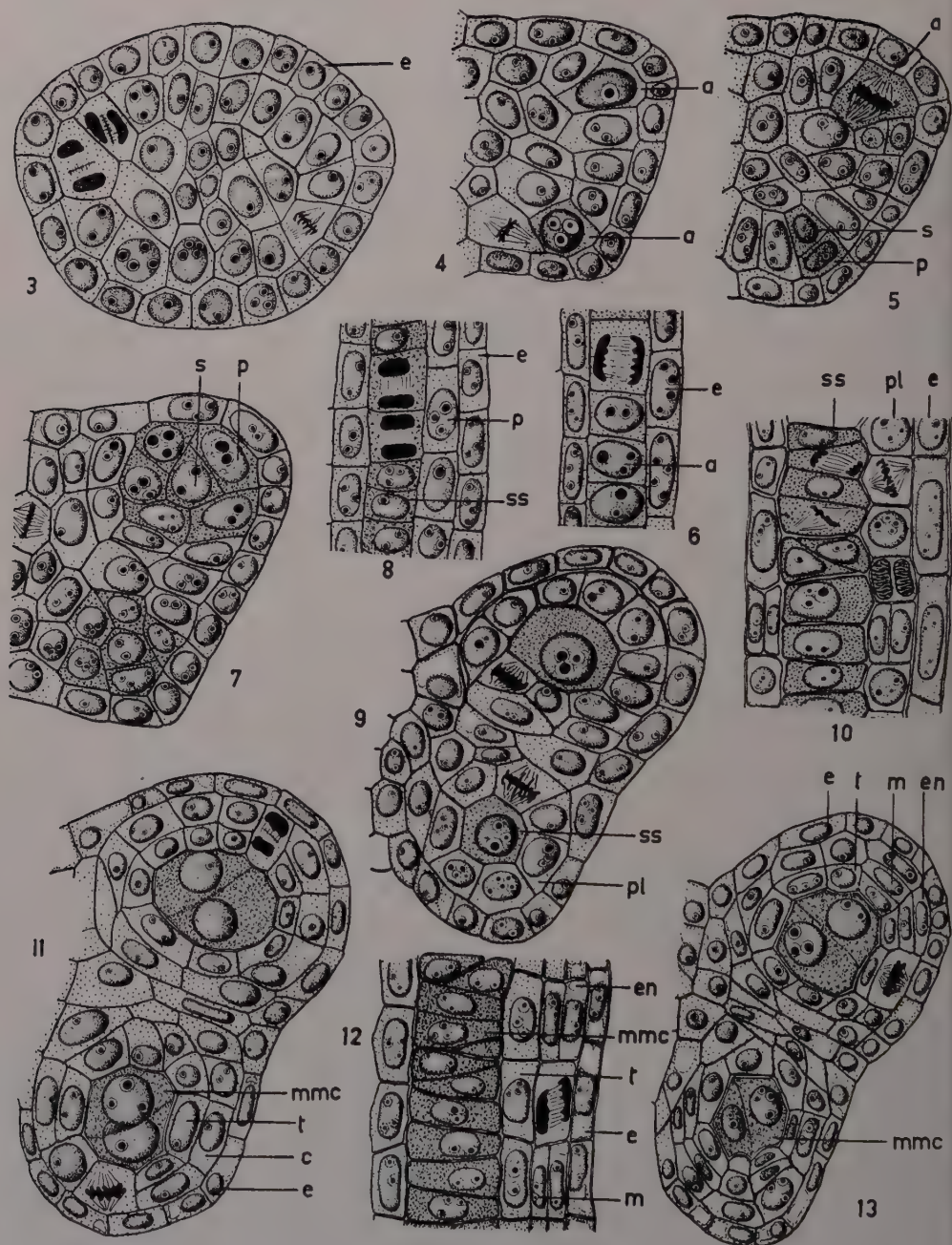
FIGS. 3-13 — The development of the anther wall and the formation of the microspore mother cells (*a*, archesporium; *c*, central layer; *e*, epidermis; *en*, endothecium; *mmc*, microspore mother cells; *m*, middle layer; *p*, primary parietal cell; *pl*, parietal layer; *s*, primary sporogenous cell; *ss*, secondary sporogenous cell; *t*, tapetum). Figs. 6, 8, 10, 12 in l.s.; remainder in t.s. Figs. 3-11 × 570. Figs. 12, 13 × 400. For detailed explanation *refer* to text.
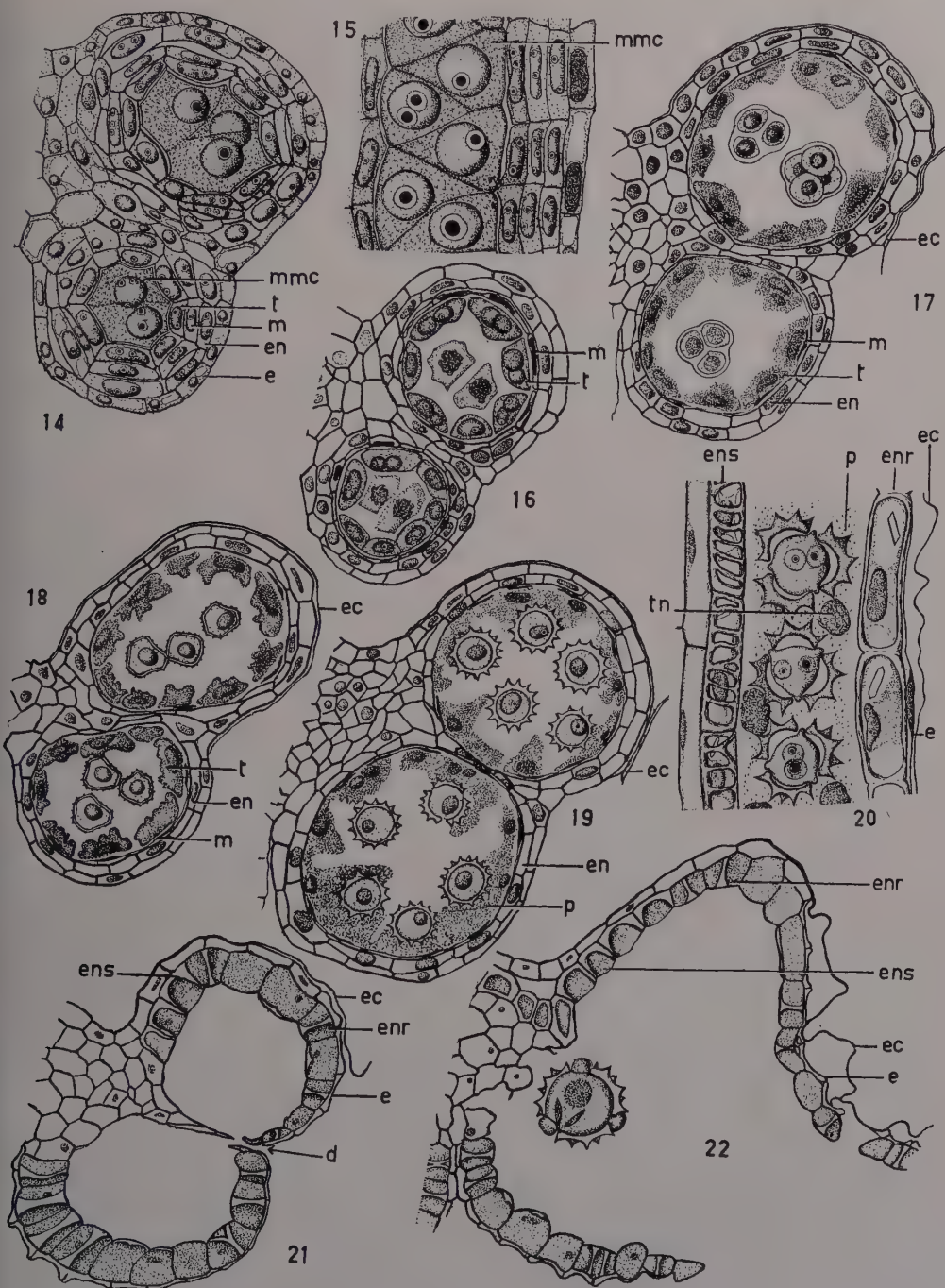
Figs. 14-22 — Changes in the anther wall leading up to the development of the periplasmodium and dehiscence (*d*, region of dehiscence; *e*, epidermis; *ec*, epidermal cuticle; *en*, endothecium; *enr*, ribbon-like thickening; *ens*, scalariform thickening; *m*, middle layer; *mmc*, microspore mother cell; *p*, periplasmodium; *t*, tapetum; *tn*, tapetal nucleus). Figs. 15, 20 in l.s.; remainder in t.s. Figs. 14, 15 × 330. Figs. 16-22 × 1000. For detailed explanation *refer* to text.

In *Podolepis*, however, two, three, or even four nuclear divisions may occur, of which the final one in each conforms to Cooper's " incomplete " type.

Witkus (1945), working on *Spinacia*, found that the tapetal nuclei divided only twice, and described three possibilities for the first division: (1) normal mitosis leading to a 2-nucleate cell; (2) normal chromosomal behaviour up to anaphase and then the formation of bridges resulting in bilobed nuclei; (3) endomitotic division. The second mitotic division was reported as being always endomitotic.

In view of the results of the present investigation, it may be that the second possibility mentioned by Witkus is really a second division.

Unfortunately, neither Cooper nor Witkus referred to the plane of the spindle in relation to any particular mitosis, so although there seems a general similarity of tapetal behaviour in *Podolepis*, it is not possible to compare the details.

In *Lactuca scariola*, Jones (1927) reported the sequence of 2, 4, 2, 1 nuclei in tapetal cells between prophase and tetrad formation, resulting from successive mitoses and nuclear fusions. Although details of these processes were not supplied, the numbers of tapetal nuclei suggest a similarity to the condition reported in *Podolepis*, which may be common in the Compositae. Support for this view comes from a number of authors who have reported nuclear fusions taking place between mitoses, but these records, although interesting, are too vague to quote individually.

MICROSPOROGENESIS — Simultaneously with the first tapetal nuclear division, the microspore mother cells round off from each other and their nuclei enter prophase (Fig. 16). Meiosis is normal and cytokinesis is of the simultaneous type, resulting from a combination of coalescing vacuoles and constriction of the common cytoplasm between the four daughter nuclei (Figs. 41-43). The microspores are arranged tetrahedrally (Figs. 44, 45). Sometimes small micronuclei are present in the tetrads, and presumably result from lagging chromosomes. There was some indication, however, of a connection between the presence of supernumerary
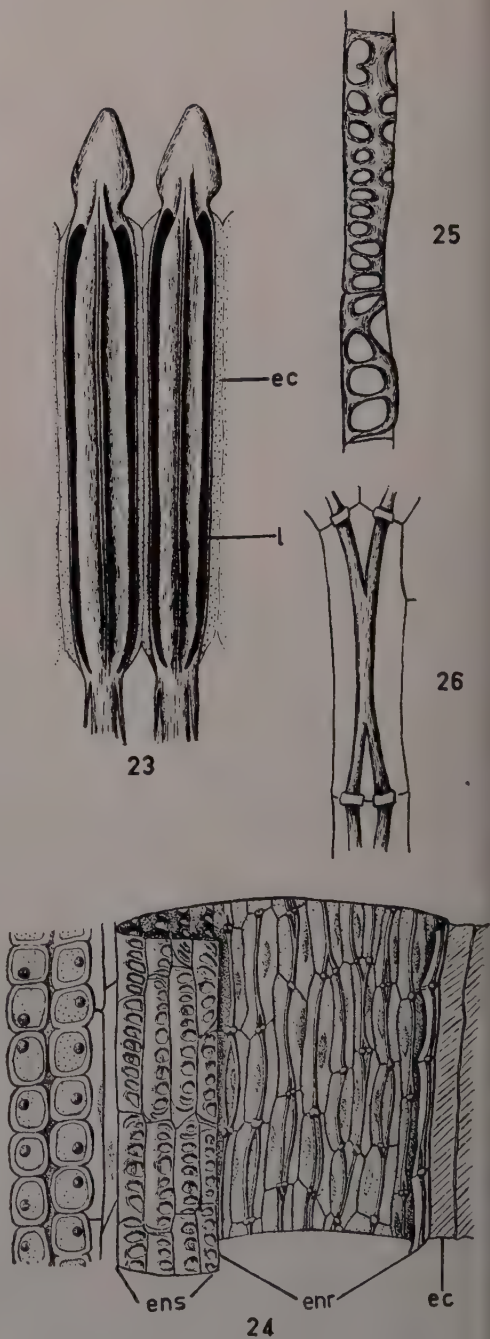
FIGS. 23-26 — Dehiscence of the anther (*ec*, epidermal cuticle; *en*, endothecium; *enr*, ribbon-like thickening; *ens*, scalariform thickening; *l*, line of dehiscence). Fig. 23. Anthers showing longitudinal dehiscence. × 23. Fig. 24. Portion of a dehisced anther in surface view, ×23. Figs. 25-26. Endothecial cells with scalariform and ribbon-like thickenings. × 670.
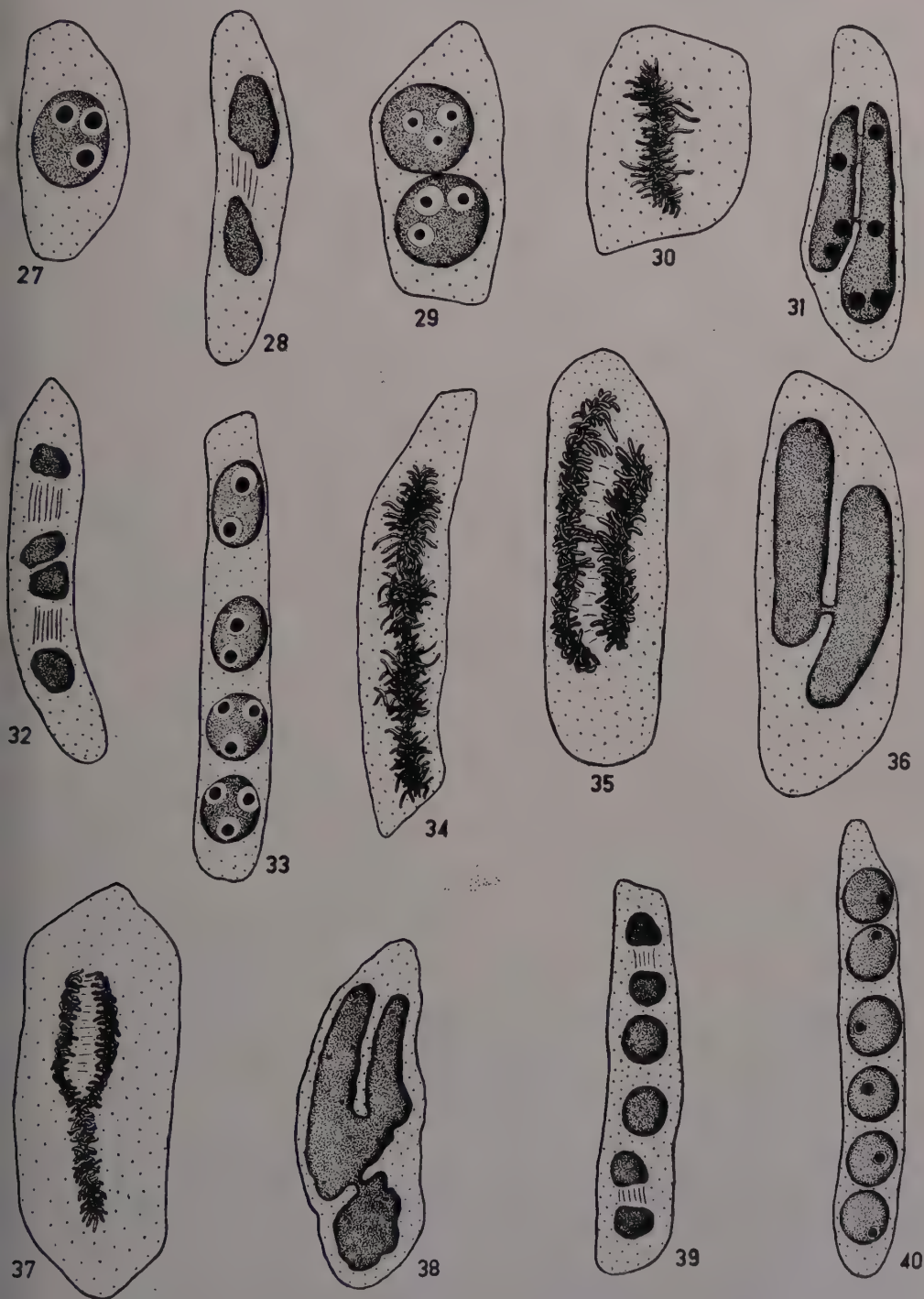
FIGS. 27-40 — Behaviour of the tapetal nuclei during meiotic prophase I. × 930.   Figs. 27-38.
P. jaceoides; Figs. 39, 40.   *P. longipedata*.

nuclei or microspores and the length of time elapsing between the collection of material and its examination. At this stage, although the tapetal cells are still in a peripheral position in the pollen sac, their walls have been digested and their cytoplasm protrudes in irregular finger-like processes (Figs. 17-19) characteristic of the amoeboid type.

The microspores, during most of their existence in the tetrad, present a rounded outline. Immediately prior to their liberation, however, minute undulations appear over their surface which mark the positions of the future spines and are the first appearance of the exine (Fig. 46). Liberation of the microspores which by now are distinctly, though minutely, spined results from breakdown of the wall of the microspore mother cell (Fig. 47). Further development of the spines proceeds rapidly prior to the formation of the periplasmodium (Figs. 18, 19) and it is doubtful, therefore, that the liberated tapetal contents fulfil any but a nutritive function.

When released from the tetrad the microspores (pollen grains) are angular, non-vacuolate and uninucleate (Fig. 48). Cytoplasmic synthesis leads to pressure on the plastic exine and the pollen grains increase in size and become rounded (Fig. 49). The exine increases in thickness and the spine becomes broader and longer. On three (occasionally four) circular and equidistant areas, however, thickening is not deposited and these persist as germ pores (Fig. 50). A central vacuole develops which displaces the nucleus and, in pressing the intine firmly against the exine, causes it slightly to protrude into the germ pores. This is the " signet-ring " configuration and in sections the intine is always slightly withdrawn from the exine due to shrinkage in processing (Fig. 51). The nucleus, occupying a position between the vacuole and the intine, is somewhat flattened on its inner side and may even be slightly saucer-shaped.

MALE GAMETOGENESIS —Gametogenesis is foreshadowed when cytoplasmic synthesis is renewed and the central vacuole of the ' signet-ring ' stage becomes considerably reduced in size. The nucleus moves away from the periphery of the cell and enters prophase (Figs. 52, 53). The plane of the spindle lies at an angle to the germ pores so that both cannot be seen in the same section. Both poles of the spindle are somewhat blunt (Fig. 54); this is more marked at anaphase, particularly at the wallward pole, and the spindle itself shows a slight asymmetry (Fig. 55). As anaphase proceeds the spindle elongates and finally extends across at least three-quarters of the diameter of the cell (Fig. 56). At telophase, however, contraction of the spindle sets in and the two daughter nuclei are brought relatively close together (Figs. 57, 58). This " stretching " of the spindle was described by Brumfield (1941) but not its subsequent contraction. Cytoplasmic cleavage then cuts off a lenticular generative cell from the large vegetative cell and the nucleoli are reformed in both nuclei (Figs. 59, 60). Although the vegetative nucleus stains less deeply than the generative one, and often loses its rounded outline, cytoplasmic synthesis is still active and leads to the elimination of the remaining small vacuoles soon after the bicelled condition of the pollen grain has been attained. The generative cell then rounds off from the intine and consists of a large deeply staining nucleus with one or more nucleoli and a thin layer of cytoplasm (Fig. 61). Although contact with the intine is lost, the generative cell remains closely associated with it and the nucleus undergoes a normal mitotic division, followed by cytoplasmic cleavage, to form two male gametes (Fig. 62). The nucleoli are not reconstituted after the division of the generative nucleus and the gametes, when first formed, are spherical, each consisting of a nucleus and a thin cytoplasmic sheath (Fig. 63). Division of the generative nucleus was seen on several occasions and the spindle was always at right angle to the intine. The shape of the gametes varies, in an apparently reversible manner, from spherical to splinter-like, and their position within the vegetative cell is entirely at random (Figs. 64, 65).

Gametogenesis is completed prior to dehiscence of the anther and the pollen grains are shed in the three-celled condition.
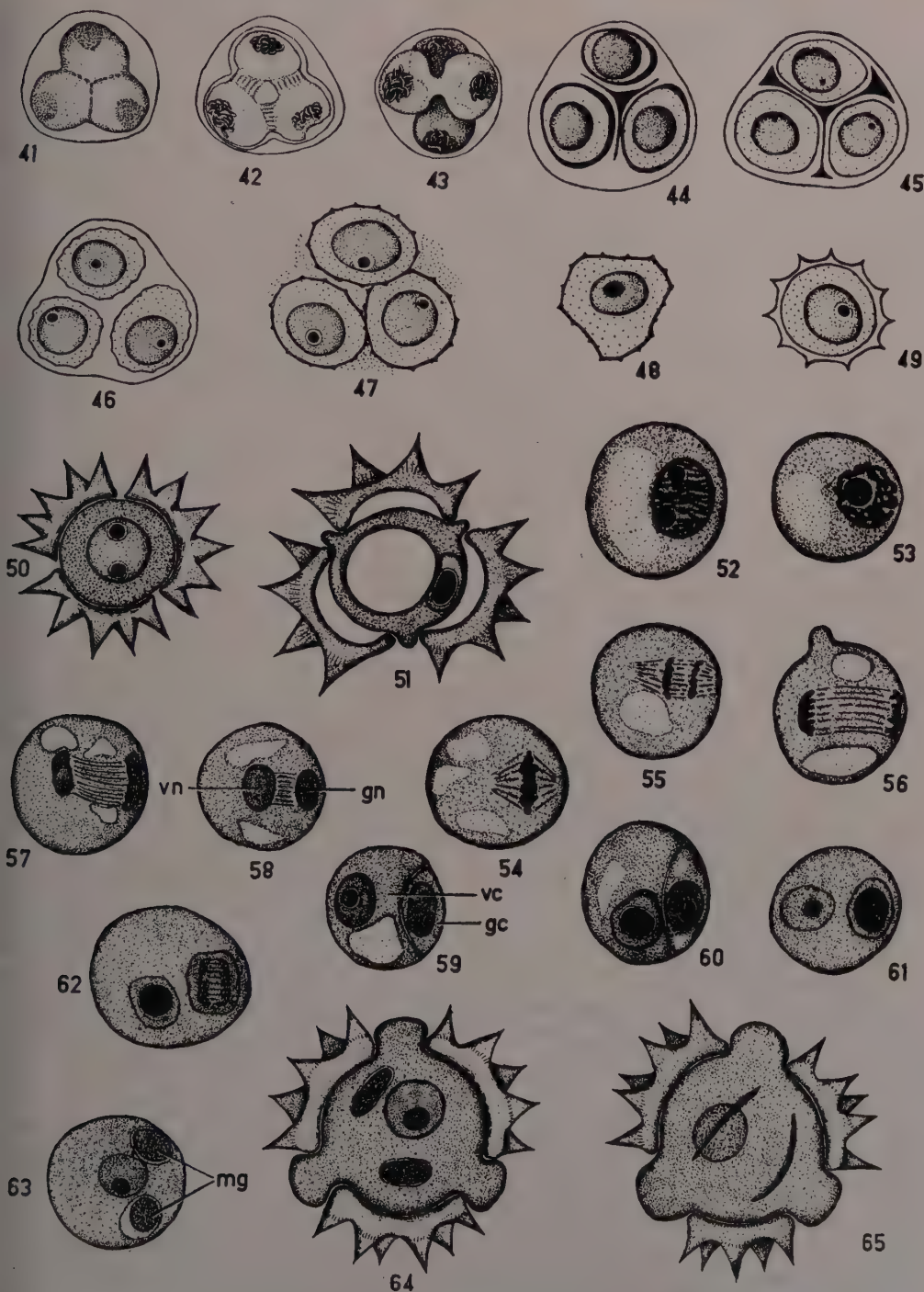
FIGS. 41-65 — Tetrad formation and male gametogenesis (*gc*, generative cell; *gn*, generative nucleus; *mg*, male gametes; *vc*, vegetative cell; *vn*, vegetative nucleus.)   In Figs. 52-63, the exine is omitted. × 930.

The stages of sporogenesis and gametogenesis are constant, within narrow limits, throughout each pollen sac and any slight variations are not related to position. Similarly, the contents of all pollen sacs of any one anther and the anthers of any one floret tend to be at the same stage of development. Minor discrepancies sometimes occur in the course of rapid processes such as post-prophase meiosis and the division of the generative nucleus.

MATURATION OF THE ANTHER WALL — During gametogenesis further changes have been taking place in the anther wall. When the pollen grains are uninucleate, the tapetal nuclei are still in position at the periphery of the pollen sac, but the cytoplasm is becoming intermingled with the pollen grains to form the periplasmodium (Fig. 19). At the binucleate stage, however, the tapetal nuclei are rather irregularly shaped and have become scattered throughout the periplasmodium (Fig. 20). They persist after the cytoplasmic constituent has been absorbed and there is no trace of any tapetal remains when the pollen grains reach maturity.

Two types of thickenings are laid down on the walls of all endothecial cells except those of the septum between adjacent pollen sacs: (1) scalariform, characteristic of cells adjacent to the anther connective (Figs. 24, 25) ; (2) ribbon-like, in the form of longitudinal bands on the inner surfaces of the transverse and tangential walls of the remaining endothecial cells (Figs. 24, 26).

Examination of both types of thickening presents considerable difficulty, and both are best seen in the dehisced anther where the endothecial cells, which line the pollen sac, can be examined directly in surface view (Fig. 24). The scalariform thickening is laid down at about the time of tapetal breakdown and it is probable that the ribbon-like bands are deposited at the same time.

DEHISCENCE — The anther dehisces introrsely by a longitudinal slit on each side at the point of junction between the two pollen sacs (Figs. 21, 22). Simultaneously, the intervening septum is torn from its attachment to the outer wall, so that the pollen sacs on each side of the anther are not united prior to dehiscence.

Both types of thickenings, by contracting at right angles to each other, play a part in the mechanics of dehiscence. The ribbon-like bands, as they dry, bring about the rupture of the endothecium and the contraction of the scalariform thickening causes the torn edges of the wall to gape and liberate the pollen into the anther tube above and at the sides of the papillate stylar arms. Contraction of the anther filaments and elongation of the style sweep the pollen out of the anther tube and separation of the stylar arms spills it over the expanded corolla lobes. Pollination, at least in the New England district, is affected by a Braconid wasp.

SEQUENCE OF DEVELOPMENT IN THE CAPITULUM — Anther maturation in the capitulum is centripetal and shows a gradient of developmental stages (Table 1).

TABLE 1 — SEQUENCE OF MICROSPOROGENESIS AND MALE GAMETOGENESIS IN THE CAPITULUM

PERIPHERAL FLORETS	MEDIAN FLORETS	CENTRAL FLORETS
Microspore mother cells in early prophase	Secondary sporocytes	Primary sporocytes
Wall of 4 layers, the middle layer breaking down	Wall of 3 layers	Wall of 2 layers
Tetrads	Early tetrads	Microspore mother cells in metaphase 1-telophase 1
1-nucleate pollen grains	Microspore mother cells in prophase	Secondary sporocytes
Signet-ring pollen grains	1-nucleate pollen grains	Tetrads
2-nucleate pollen grains	Signet-ring pollen grains	Tetrads
Gametes	2-nucleate pollen grains	Signet-ring pollen grains

The chief variation from the standard pattern in all the species examined was in the meiotic stages other than prophase, since these are passed through rapidly. As a result, when the anthers of the peripheral disc florets contain mature tetrads, microspore mother cells from metaphase to telophase will be found in the central florets.

Dehiscence follows closely the formation of male gametes and pollen is shed from the anthers of the peripheral florets when the pollen grains of the central florets are at the late signet-ring stage. The normal development of the latter, however, is not arrested or affected in any way by pollen maturation in the outer florets, and there is no evidence of a male fertility gradient across the capitulum.

Summary

Microsporogenesis and male gametogenesis are identical in *Podolepis jaceoides*, *P. canescens*, *P. longipedata*, *P. neglecta* and *P. arachnoidea* and these processes conform to the general pattern described by Maheshwari (1949, 1950). The young anthers are free from each other and their apparent fusion at maturity results from the adhesion of epidermal cuticle on adjacent anthers. The wall of the pollen sac is composed of four layers, whose origin and fate are traced. The endothecial cells develop two types of thickening and the role of each in dehiscence is described. The behaviour of the tapetal nuclei during meiotic prophase I is described and a difference was found between their behaviour in *P. jaceoides* and the other species examined. A series of tapetal mitoses is passed through in which the orientation of the spindle follows a definite pattern. No fusion of nuclei, as such, was found, but coalescence of separate spindles at the final tapetal mitosis results in a lobed nucleus with varying degrees of polyploidy. In mature tapetal cells the binucleate condition was found to be the exception rather than the rule. Tetrads are of the tetrahedral type and the microspores develop minute undulations on their surfaces immediately prior to their liberation from the tetrads. These are the first indications of the spines which cover the exine of the mature pollen grains. The spindle of the first pollen grain mitosis is symmetrical when first formed but becomes asymmetrical as metaphase proceeds, the wallward pole becoming short and blunt. Elongation of the spindle takes place at anaphase and subsequent contraction results in the daughter nuclei being brought close together at telophase. Cytoplasmic cleavage cuts off a lenticular generative cell from the vegetative cell, and by rounding off this becomes detached from the intine and enclosed in the vegetative cytoplasm. A normal mitosis of the generative cell gives rise to the male gametes and the pollen grains are shed in the three-celled condition.

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SHOOT PRODUCTION IN CULTIVATED TEA (*CAMELLIA SINENSIS* L.) — II. THE BRANCH SYSTEM

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Introduction

Growth induced by plucking a tea bush (*Camellia sinensis* L.) is essentially sympodial in nature and the yield of shoots harvested for the manufacture of the commercial product is heterogenous in composition. For these reasons, knowledge of the morphology of the complex system of branches at the plucking surface, rate and time of development of the various branches and sub-branches, and composition of the harvested shoots and its seasonal variation are prerequisites for an understanding of the process of shoot production by plucked tea bushes.

The first paper of this series (Barua & Wight, 1959) discussed the effects of variation of tipping height on top-growth, radial growth, and yield of plucked shoots. The same experiment was utilised for observing the nature of the shoot system, its development and composition. This paper summarises the information contained in those observations.

Design of the Experiment

Details of the experimental design are given in the earlier paper (Barua & Wight, 1959). Fifteen-year old bushes of a clone (16/10/22), representative of much of the tea grown in north-east India, were tipped and plucked at heights of 5, 10, 15, 20, 25, 30 and 35 cm. The bushes in the eighth treatment were neither tipped nor plucked. The first tipping was done on 3rd March, subsequent plucking being done every fifth day. Plucking ceased on 22nd December.

Shoots with more than one leaf and all dormant (*banjhi*) shoots were removed at the plucking level or at the uppermost cataphyll if that was above the plucking level. When the first cataphyll was much above the plucking level, then the piece of projecting stem was broken back to the cataphyll nearest to the plucking surface. This is usual and was done to maintain a flat plucking surface and prevent its excessive rise. This procedure caused a gradual rise of the plucking surface of the order of 5 cm in one season.

In addition to the observations recorded in the earlier paper, plucked shoots were categorized into types, the weight and number of each type and of the broken back pieces of stems being recorded separately at every plucking round. Ten primaries on each bush, which had been tipped before 20th April, were marked diagonally across each bush and the number of successive orders of lateral shoots developing on these primaries was counted at intervals of approximately 15 days. The total number of lateral shoots of the different orders was counted subsequently for each bush from the prunings stored in the laboratory.

Composition and Seasonal Changes in Yield

The contribution by shoots with one, two and three leaves, and pieces of stems broken back to the plucking level (designated 1 + b, 2 + b, 3 + b, and *janams*, respectively) towards total weight of plucking (yield) is shown in Table 1. Under conditions of this experiment approximately 80 per cent of the yield at all tipping heights was made by shoots with two leaves. The proportion of shoots with one leaf decreased as the tipping height was gradually raised from 5 to 35 cm. The same amount of breaking back had to be done at all tipping heights.

TABLE 1 — PERCENTAGE OF 1+b, 2+b AND 3+b SHOOTS AND *JANAM* IN YIELD, SHOWN IN THE LAST COLUMN

TIPPING HEIGHT	PERCENTAGE OF YIELD				YIELD PER BUSH IN kg
	1+b	2+b	3+b	*Janams*	
5 cm	2·7	78·9	4·5	13·9	2·24
10 cm	2·2	79·7	6·0	12·1	2·46
15 cm	2·4	79·8	5·2	12·6	2·91
20 cm	1·6	80·7	5·8	11·9	2·48
25 cm	1·5	79·5	5·4	13·6	2·66
30 cm	1·5	78·0	7·0	13·5	2·53
35 cm	1·3	78·7	6·4	13·6	2·08

Each type of shoot in Table 1 was sub-divided into growing and dormant categories, depending on the state of growth or dormancy of the apex (Wight, 1955). The proportion of dormant shoots decreased, and growing shoots increased with the rise of the tipping height (Table 2). This is more clearly seen from the ratios of dormant to growing shoots shown in the last column of Table 2.

In north-east India, the most valuable teas (commonly known as 'second flush' tea) are made from about the middle of April till the end of June. Tea of good quality can also be made during the autumn, provided weather conditions remain favourable. Tea made during the rains (July-September), amounting to a half of the total yield, is comparatively of poor quality. Different tipping treatments altered the proportion of crop harvested at these times of the year (Table 3). The second column of Table 3 can be regarded as representing the percentages of 'second flush' yield in different tipping treatments since less than one per cent of the total yield was harvested before mid April. The 'second flush' yield dropped by 15 per cent when the tipping height was raised from 5 to 35 cm. There was a reversal of this trend during and after the rains.

SHOOT WEIGHT — The average weight of a shoot was calculated from the records of weight and number kept separately for each shoot type. The changes in shoot weight with tipping height and time of the year were examined in terms of

growing shoots with two leaves, these constituting two-thirds of the total yield. Analysis of variance of the data presented in Table 4 was based on average shoot weights for three consecutive pluckings (periods). Shoot weight increased gradually with the rise of the tipping height, the linear component of treatment variance being highly significant. The increase in the average shoot weight from 5 to 35 cm tipping heights is approximately 30 per cent (Table 5).

Changes in shoot weight with time are very highly significant and the linear component of the variance due to period is also significant at the 1 per cent level (Table 4). The interaction of treatment with period is not significant. In the absence of this interaction, data presented in Fig. 1 are in terms of the means for the seven tipping treatments. Figure 1 shows that the weight of a standard 2 + b growing shoot was at a maximum in the early part of the plucking season and dropped

TABLE 2 — PROPORTION BY WEIGHT OF GROWING AND DORMANT SHOOTS IN YIELD

TIPPING HEIGHT	PERCENTAGE OF YIELD		DORMANT/ GROWING
	Growing shoots	Dormant shoots	
5 cm	68·6	17·5	0·26
10 cm	72·0	15·9	0·22
15 cm	71·6	15·8	0·22
20 cm	73·5	14·6	0·20
25 cm	72·8	13·7	0·19
30 cm	72·6	14·0	0·19
35 cm	74·1	12·3	0·16

TABLE 3 — YIELD AT DIFFERENT TIMES OF THE YEAR EXPRESSED AS PERCENTAGE OF THE TOTAL

TIPPING HEIGHT	PERCENTAGE OF YIELD		
	Till end June	July-September	From October
5 cm	34·0	43·0	23·0
10 cm	28·9	46·8	24·8
15 cm	25·7	47·2	27·1
20 cm	24·9	47·7	27·4
25 cm	21·2	47·9	30·9
30 cm	21·9	49·3	28·7
35 cm	19·4	51·2	29·4

TABLE 4 — ANALYSIS OF VARIANCE OF THE WEIGHT OF A GROWING SHOOT WITH TWO LEAVES, AFTER GROUPING OF THE DATA INTO PERIODS OF THREE CONSECUTIVE PLUCKING ROUNDS

Source	D.F.	Mean Square
Repeat	2	0·0085
Tipping height (H)		
Linear	1	0·5608***
Residual	5	0·0113
Error	12	0·0072
Period (P)		
Linear	1	4·2316**
Residual	20	0·5044
H × P	126	0·0127
Error	294	0·0704
Total	461	

Two and three asterisks indicate significance at 1·0 per cent and 0·1 per cent levels, respectively.

TABLE 5 — WEIGHT OF A GROWING SHOOT WITH TWO LEAVES AT DIFFERENT TIPPING HEIGHTS, IN TERMS OF AVERAGE FOR THE WHOLE PLUCKING SEASON

Tipping height	Shoot weight in gm
5 cm	0·387
10 cm	0·389
15 cm	0·436
20 cm	0·429
25 cm	0·467
30 cm	0·477
35 cm	0·507

to a half by early September, and to a fifth by the end of the year. Thus changes in shoot weight associated with tipping height (Table 5) are superimposed on more profound seasonal changes. It should be noted, however, that the branch system on a plucked tea bush gets increasingly complex with the progress of the season. The changes in shoot weight with time cannot, therefore, be dissociated from the increasing complexity of branching at the plucking surface, the nature of which is discussed elsewhere in this paper. Similar results are obtained by taking the weight of a dormant shoot, the average weight of which is approximately half
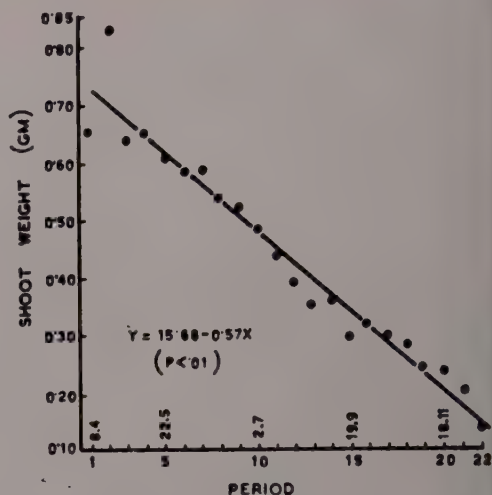
Fig. 1 — Changes in the weight of a growing plucked " two and a bud " shoot with time. Each point is the mean shoot weight of three consecutive plucking rounds (period). The mean date of every fifth period is indicated in the figure. Data in terms of averages for the seven tipping treatments.
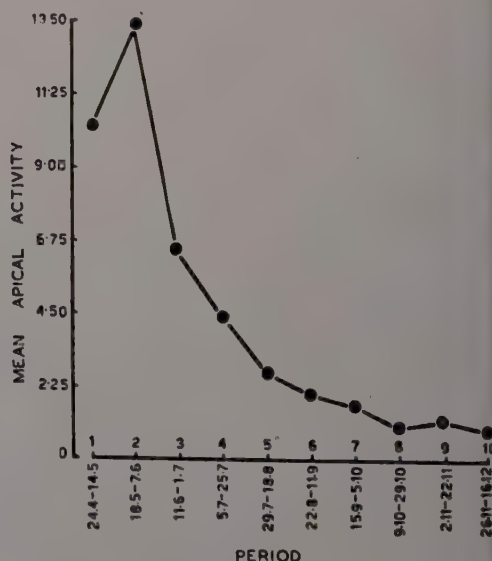
Fig. 2 — Changes in apical activity along time. Each point is the mean of six consecutive plucking rounds (period). Data in terms of averages for the seven tipping treatments.

that of a growing shoot with equal number of leaves.
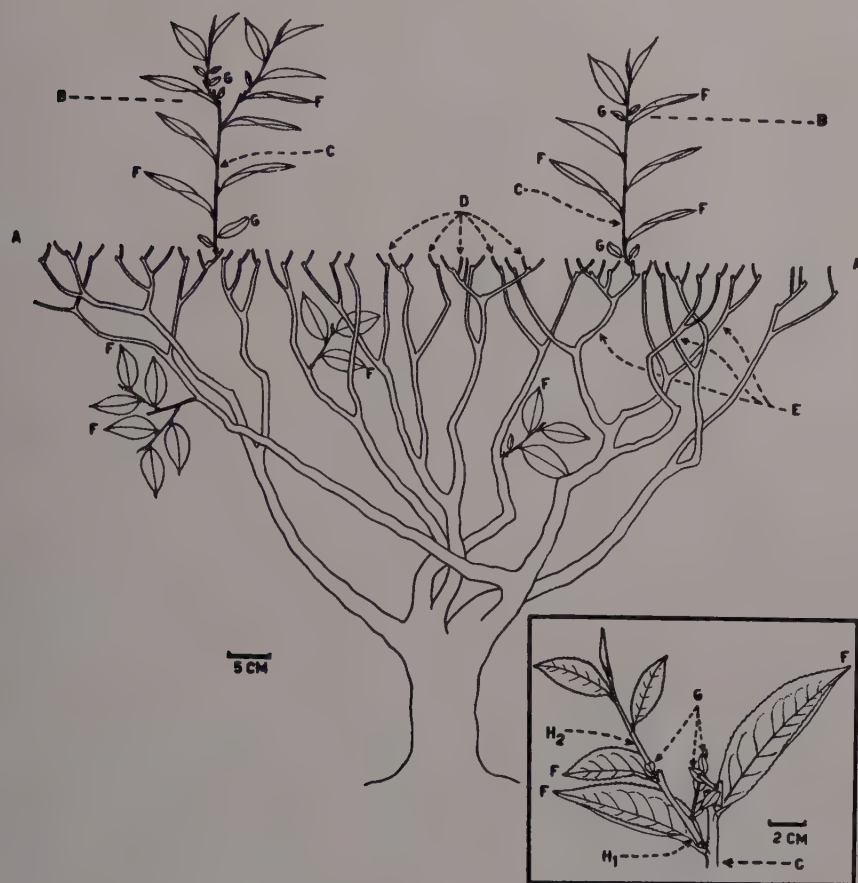
Fig. 3 — Diagrammatic representation of a pruned and plucked tea bush (A-A, pruning level; B-B, tipping level; A-B, first flush; C, primaries; D, stubs; E, sticks; F, maintenance leaves; G, cataphylls; H_1 and H_2 of the inset refer to first and second order laterals, respectively.

APICAL ACTIVITY — It is defined as the reciprocal of *dormancy index* (Barua & Wight, 1959), the mean apical activity being increased with the height of tipping, except when tipping is done at the first horizon of dormancy. Further analysis of the data on the lines of shoot weight (Table 4) shows a significant fall in apical activity with the progress of the plucking season. The interaction of tipping height with time of the year is not significant. The time trend of apical activity shown in Fig. 2 is, therefore, based on the means of all tipping heights.

The dormancy indices of plucked and unplucked bushes fluctuate throughout the year. Some indications of this are given by Wight & Barua (1955). While similar fluctuations were observed in this experiment, the purpose here is to demonstrate the overall time trend of apical activity. This is achieved in Fig. 2 by averaging the apical activity values for every six consecutive plucking rounds.

Development of the Shoot System

The structure of a plucked tea bush, described in the earlier paper, and the initial stages of development of its shoot system at the plucking surface are represented diagrammatically in Fig. 3. Decapitation (tipping) of a primary causes the axillary buds immediately below the

point of tipping to throw out lateral shoots of the first order. When the first order shoots are plucked leaving on the bush short pieces of stem with the basal cataphylls, then buds on the axils of the cataphylls produce shoots of the second order. The second order shoots when plucked in their turn give rise to shoots of the third order and so on. The process is repeated till the end of the plucking season with the emergence of higher and higher orders of lateral shoot.

THE FIRST ORDER LATERAL — Every leaf on a primary could subtend a lateral shoot of the first order, but the actual number of laterals did not increase in the same proportion as the number of leaves on a primary. The number of axillary buds on a primary which failed to grow increased gradually as the tipping height was raised from 5 to 35 cm. These results are shown in Table 6.

Data in Table 6 show that the development of axillary buds into first order laterals was limited to an average of four leaves below the point of tipping. Some buds on the axils of the fifth, sixth and lower leaves of high-tipped primaries produced laterals of the first order, but such buds were very few. In general, the percentage of active, axillary buds on a primary varied inversely as the distance from the point of tipping, the number of active buds decreasing from 100 per cent on the axil of the first leaf to less than 5 per cent on the seventh leaf. Only a negligible number of axil buds on the eighth and lower leaves developed into
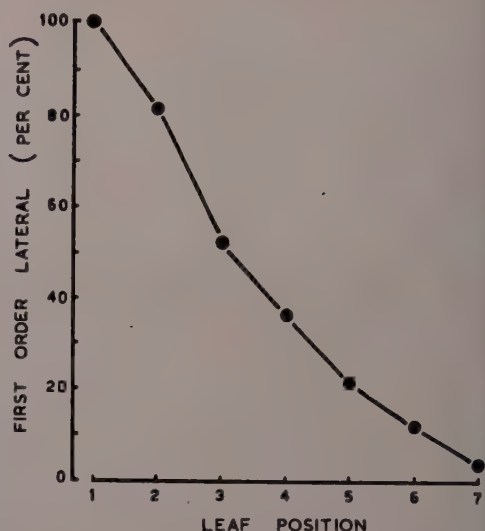
FIG. 4 — Percentage of leaf-axil buds at successive positions on primaries producing first order laterals. Leaves are numbered downwards from the point of tipping, the leaf in position 1 being nearest to the tipping level. Data in terms of averages for the seven tipping treatments.

lateral shoots. These results are shown graphically in Fig. 4 in terms of the means for the seven tipping heights.

The total number of shoots borne by each first order lateral was counted with a view to obtain a measure of the contribution made by successive first order laterals towards yield. The count was based on all the shoots on a bush. The count shows that the number of shoots borne by the uppermost first order lateral was double the number borne by the second. The number of shoots borne by the second, third and other first order laterals at tipping heights of 15 cm and above showed a tendency to decrease with increasing distance of the leaf from the point of tipping. Raising the tipping height from 5 to 20 cm resulted in an increase in the number of shoots borne by every first order lateral at corresponding positions on the primary; any difference in height above 20 cm ceased to have noticeable effect. These results are illustrated in Fig. 5. For the sake of clarity, data for 5, 20 and 35 cm tipping heights only are presented in the figure.

TABLE 6 — AVERAGE NUMBER OF LEAVES, FIRST ORDER LATERALS AND PER CENT ACTIVE BUDS PER PRIMARY

TIPPING HEIGHT	NUMBER OF LEAVES	NUMBER OF FIRST ORDER LATERALS	PER CENT ACTIVE BUDS
5 cm	2·73	1·94	71·1
10 cm	3·78	2·46	65·1
15 cm	5·00	2·68	53·6
20 cm	5·80	2·85	49·1
25 cm	6·94	3·07	44·2
30 cm	7·60	3·18	41·8
35 cm	8·52	3·43	40·2
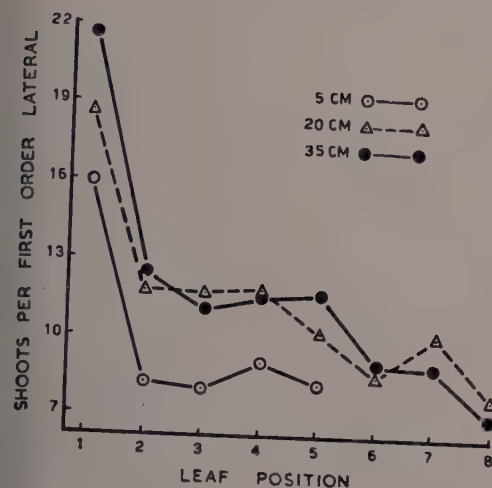
For every shoot of the first order there were, on the average, 2·06 shoots of the second order. If this rate were maintained, there ought to have been 4 shoots of the third order, 8 of fourth order and so on. The relative frequency figures of Table 8, however, show that the rate of multiplication of shoots at the plucking table from successive orders of laterals dropped in the order 2·0, 1·5, 1·2, 0·8, 0·4, 0·2 and 0·1 in the course of the growing season. The number of buds which failed to grow went on increasing and some of these buds died before the end of the plucking season.

RATE OF REGENERATION — It is of interest to examine here the rate of regeneration of a leaf-axil bud under the

NUMBER OF SHOOTS — The tipping treatments significantly altered the total number of shoots plucked from a bush (Table 7). The increase in the weight of shoots plucked off a primary with gradual rise of the tipping height has been recorded in the earlier paper (Barua & Wight, 1959). The number of shoots borne by a primary increased rapidly between 5 and 15 cm tipping heights, the increase with further rise of the tipping height being non-significant. The number of first order laterals showed the opposite trend (Table 7).

ORDER OF LATERAL — A maximum of eight orders of lateral appeared in the course of the plucking season. Considering in terms of the average for the whole bush, there was no appreciable difference in the frequency per cent of shoots of different orders between 10 and 35 cm tipping heights, which was somewhat different from the frequencies at the 5 cm height of tipping. The mean per cent frequencies at 5 cm are shown separately in Table 8. The relative frequencies shown in the last column of the table are based on the averages for all seven tipping heights.

TABLE 7 — TOTAL NUMBER OF SHOOTS AND NUMBER OF FIRST ORDER LATERALS AT DIFFERENT TIPPING HEIGHTS ADJUSTED ON THE NUMBER OF STICKS

TIPPING HEIGHT	TOTAL SHOOT NUMBER		NUMBER FIRST ORDER LATERALS PER BUSH
	Per bush	Per primary	
5 cm	5091	23	444
10 cm	5794	32	436
15 cm	6509	41	432
20 cm	5959	43	389
25 cm	5659	42	409
30 cm	5265	45	377
35 cm	4069	46	310
L.S.D. at P = 0·05	138	6	72

TABLE 8 — FREQUENCY PER CENT OF SHOOTS OF DIFFERENT ORDERS AND RELATIVE FREQUENCIES

ORDER OF SHOOT	FREQUENCY PER CENT		RELATIVE FREQUENCY ON AVERAGE
	Average of all tipping heights	At 5 cm height	
1st order	7·3	8·3	1·00
2nd order	15·0	16·4	2·06
3rd order	22·1	23·0	3·03
4th order	25·5	25·2	3·49
5th order	19·7	18·1	2·70
6th order	8·5	7·6	1·17
7th order	1·7	1·3	0·23
8th order	0·1	0·1	0·02

influence of repeated plucking. Other
things being equal, regeneration can be
affected by time of tipping and position
of the buds on the primary. The question
can, therefore, be examined either on
particular buds at specified positions or on
all buds on the bush as a whole. The
former is not related to yield but can be
supposed to give a truer picture of the
regeneration phenomenon than the latter,
which reflects the actual state of the bush
in the field. Both these aspects were
examined.

The number of shoots produced during
intervals of approximately fifteen days by
the topmost first order lateral on thirty
primaries in each tipping height, ten in
each bush, is shown in Fig. 6. These
primaries, which were tipped before 20th
April, can be considered comparatively
more vigorous than many other primaries
on the same bush. Data in Fig. 6 for the
three tipping heights of 5, 20 and 35 cm
indicate the general pattern of regeneration
at the other tipping heights. The periodic

Fig. 7 — Total number of shoots plucked from
a bush on three consecutive plucking rounds.
Data for 5, 20 and 35 cm tipping heights.

Fig. 6 — Frequency of shoots formed during
intervals of approximately 15 days on the up-
permost first order lateral of thirty marked
primaries. Data for 5, 20 and 35 cm tipping
heights.

fluctuations in the graphs are possibly
related to the times of formation of
different orders of lateral shoots (see
below). Underlying these fluctuations
is a steady time trend of increasing shoot
number at each tipping height.

In terms of averages for the whole bush,
data corresponding to Fig. 6 are illustrated
graphically in Fig. 7. Decreasing shoot
number from early October till the end of
the plucking season is the main difference
between Fig. 6 and Fig. 7. This steady
decrease shows that the accumulation of
non-growing buds outstripped the incre-
ments of new shoots on the topmost first
order laterals.

Time of Emergence of Laterals

Time of emergence of successive orders
of lateral was observed on ten primaries
marked diagonally across each bush.
The primaries were tipped before 20th
April, but not all on the same day. In
bushes plucked at 5 and 10 cm heights,
decapitation of all marked primaries was
complete before mid-March, while in
bushes plucked at 25, 30 and 35 cm, some
of the selected primaries reached the
tipping level as late as mid-April. Be-
tween tipping of the first and the last

marked shoots, there was an interval of approximately a month and a half. This was unavoidable.

Counts of the number of laterals of different orders developing from successive leaf-axils on the marked primaries were made at intervals of approximately 15 days. However, the interval between the last and the penultimate observations increased to 32 days, as one count had to be sacrificed in favour of other pressing observations. All the laterals, whether plucked or not, were counted at each observation, the number developing in any one interval being obtained from a difference between two consecutive counts. The first and the last counts were taken on 21st April and 5th December, respectively.

Some of the first order laterals at all tipping levels, and second order laterals

at the higher tipping heights emerged before the beginning of observations. The dates of emergence of these two orders are not, therefore, known. The time of emergence of the remaining orders of lateral and the number of each order observed on successive dates are shown graphically for 5, 20 and 35 cm tipping heights in Fig. 8. Data for other tipping heights are not included in Fig. 8 as the curves are the same for tipping heights of 15 cm and above, and different from those of 5 and 10 cm. There is no difference between these latter two heights of tipping.

Figure 8 shows that the formation of the second, third, fourth and higher orders of lateral was delayed at the lower tipping heights, despite earlier decapitation of the primaries on low-tipped bushes. The 5 cm height of tipping was lagging behind 20 cm and 35 cm heights by one order of lateral.

Data presented in Fig. 8 are based on the uppermost axillary bud on a primary as lateral formation at all tipping heights was progressively delayed with increasing distance of the axillary bud from the point of tipping. Times of formation of the third and fourth order laterals from successive axillary buds on a primary are taken as examples to illustrate this latter point (Table 9).

Discussion

Variation of tipping height altered the composition and seasonal distribution of yield. The weight of shoots plucked during the early part of the year gradually increased as the tipping height was reduced from 35 to 5 cm. Increase in the valuable ' second flush ' yield is the main argument in favour of low tipping. The advantage of an early increase in yield should, however, be weighed against the presence of higher proportion of dormant (banjhi) shoots and the fall in apical activity associated with low tipping. The former is generally believed to reduce the market value of a tea and the latter was shown to be a measure of loss of vigour (Barua & Wight, 1959). In the same paper, plucking at the first modal height of dormancy was suggested to be a suitable compromise, and data presented
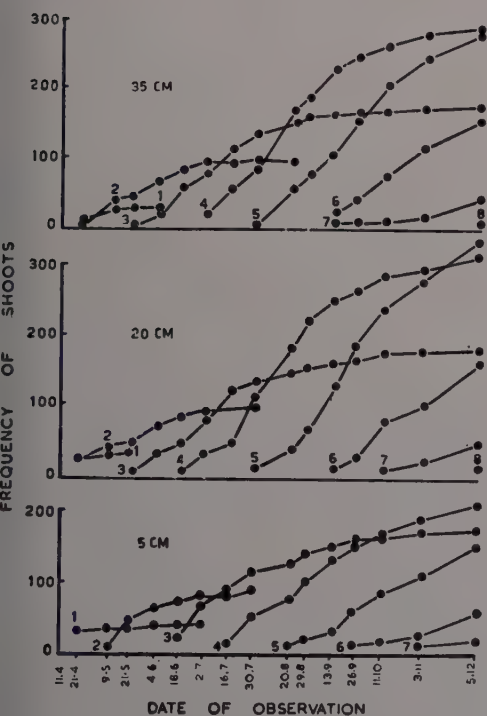
Fig. 8 — Frequency of laterals of different orders developing from the uppermost axil bud on thirty marked primaries at successive dates of observation. Orders of lateral are indicated by numbers 1 to 8. Data for 5, 20 and 35 cm tipping heights.

TABLE 9 — INTERVALS DURING WHICH THE THIRD AND FOURTH ORDER LATERALS FROM SUCCESSIVE LEAF-AXIL BUDS ON PRIMARIES WERE RECORDED FOR THE FIRST TIME AT DIFFERENT TIPPING HEIGHTS. MEAN DATES OF OBSERVATION ARE SHOWN IN ITALICS

POSITION OF AXIL BUD ON PRIMARY	TIME INTERVAL	
	Third order	Fourth order
1st (Uppermost)	21 May-18 June *5 June*	4 June-16 July *27 June*
2nd	4 June-2 July *19 June*	2 July-30 July *16 July*
3rd	18 June-16 July *3 July*	16 July-20 August *3 August*
4th	2 July-20 August *27 July*	20 August-26 September *8 September*
5th	16 July-13 September *14 August*	29 August-11 October *19 September*

here do not give any reason to suppose that better results might be obtained by plucking at a different height.

The time trends of decreasing apical activity and shoot weight (Figs. 1, 2) cannot, however, be attributed to gradual loss of vigour of the bush or to deteriorating climatic conditions, although some deleterious effects of adverse climatic conditions obtaining towards the end of the plucking season cannot be entirely ruled out. Portsmouth (1957) at St. Coombs observed a correlation in the second and third year from pruning between rainfall 1-3 months before plucking and flush (growing) shoot weight. No such correlation has been observed in our present experiment. It should, however, be noted that climatic and cultural conditions differ widely between St. Coombs and Tocklai. The latter has a well-marked cold, dry season from October to March. Tea bushes are pruned every year during this season reducing thereby the loss of yield to a minimum. St. Coombs does not have a similar season and plucking is continued throughout the year, pruning being done every 3-4 years.

The decrease in the weight of a growing shoot with age from pruning has been recorded by Tubbs (1936) and also by Portsmouth (1957). Internal competition among increased number of shoots for materials used in growth was suggested by Tubbs to be the most important factor affecting shoot weight. On the other hand Portsmouth laid emphasis on the increasing complexity of branching at the plucking surface, implying thereby interference with the movement of water and nutrients to the growing apices. Wight (1955) draws attention to the possibility of a loss of vigour intrinsically due to ageing of the apical meristems, which might need to be considered separately from effects due to the branch system. He points out that, following such a loss of vigour, there may be an inherent necessity for a bud to pass through a relatively prolonged dormant period (longer than the period of minimal rest). The same author suggests that the effect of pruning, as distinct from the effect of plucking, is a result of the growth of buds which have, in fact, been through one of these long periods of dormancy.

It should be pointed out that size and weight of shoots vary between clones and populations. External factors might alter within limits the average shoot weight of a population or clone as demonstrated by the variation of tipping height in the present experiment (Table 5). However, the downward trend of shoot weight with age from pruning or, in annually pruned bushes, from tipping persists in spite of favourable external conditions, which operate to raise the overall level of shoot weight at any instant of time.

Particular significance attaches to the first order laterals from which the higher orders develop. The average number of first order laterals on a primary was not increased beyond a certain maximum by decapitating a long length of primary, causing thereby more axillary buds to be left below tipping. Data in Table 6 indicate that the stimulus of tipping does not extend beyond three to four leaves below the point of tipping. The tendency to yield a constant number of plucked shoots per primary at tipping heights of 15 cm and above (Table 7) is thus explicable from these results.

On a tipped primary, the uppermost first order lateral was the most vigorous. This is in complete accord with the observations of Cohen Stuart (1919). Shoots borne by the topmost first order laterals contributed 40 to 64 per cent of the total yield. The yield from the second, third and other first order laterals decreased in proportion to the distance of the lateral from the point of tipping (Table 10). The few first order laterals which developed from the axils of the sixth, seventh and lower leaves (Fig. 4) made an insignificant contribution towards yield. This is particularly so at the lower tipping heights (Table 10) where their number was even less. The number of leaves on a plucked bush cannot, however, be reduced to the extent of leaving an average of three to four axillary buds per primary, without causing an insufficiency of maintenance leaves. The increase in the number of maintenance leaves per primary, which was shown to be strictly proportional to the height of tipping (Barua & Wight, 1959), is associated with gradual increase in yield of the third, fourth and other first order laterals lower down on the primary (Table 10). A deficit of maintenance leaves at the lower tipping heights can be supposed to cause loss of vigour, and this is reflected in the decrease of individual shoot weight and apical activity, and drop in average shoot number per first order lateral (Fig. 5).

TABLE 10 — PERCENTAGE CONTRIBUTION TOWARDS TOTAL YIELD OF A BUSH BY SHOOTS ON FIRST ORDER LATERALS AT SUCCESSIVE POSITIONS ON A PRIMARY. DATA FOR 5, 15, 25 AND 35 CM TIPPING HEIGHTS

POSITION OF FIRST ORDER LATERAL	YIELD PER CENT			
	5 cm	15 cm	25 cm	35 cm
1 (Uppermost)	64·5	52·1	44·4	40·9
2	22·3	25·0	24·0	21·5
3	8·6	12·3	14·7	15·3
4	3·5	6·8	9·2	11·2
5	1·0	2·7	5·0	6·3
6	—	0·8	1·7	2·7
7	—	0·1	0·6	1·2
8 and lower	—	—	0·3	0·9

In the present trial, fourth order laterals yielded the maximum number of plucked shoots at all tipping heights, although low tipping at 5 cm and 10 cm reduced by one order the number of orders of lateral produced in the course of a plucking season (Fig. 8). On the day when tipping was done for the first time (3rd March), more growth was removed from the low-tipped bushes, and many shoots on these bushes had to be broken back to hard wood. Less orders of lateral on the low-tipped bushes can be associated with the age of the stem, axillary buds on which were forced to grow, or with the reduced number of maintenance leaves. The latter seems to be a more plausible reason, as low tipping does not appear to have delayed development of the first order laterals. The development of the second and third order laterals was delayed at 5 cm and 10 cm tipping heights, due possibly to insufficiency of materials required for growth. The results emphasise the need for maintaining an adequate cover of foliage on the bush to sustain production of crops of shoots.

Considering only the uppermost first order laterals on the marked primaries, all of which were tipped early in the season, it is observed that each first, second and third order lateral gave rise to 3·0, 1·8 and 1·5 laterals of the second, third and fourth order, respectively. The rate of multiplication of shoots went on diminishing with increasing order of branching. Figure 8 shows that there could have been more shoots of the fifth, sixth and higher orders if plucking were to continue for some more time, and that continuance of plucking would hardly have affected the number of third and fourth order laterals. The data for the latter two orders of lateral lead to the conclusion that the number of those buds on the stubs of plucked shoots, which fail to grow, increases with the order of branching. Examination of the plucking surface shows that many of these non-growing buds die before the end of plucking.

Shoot weight decreased steadily from the beginning of the plucking season (Fig. 1) while the number of shoots per bush increased till early October (Fig. 7).

Yield, which is the product of these two, reached the maximum in late August or early September. After shoot number started falling from early October, yield decreased at a rapid rate.

The foregoing considerations suggest that yield increase brought about by cultural and environmental factors over a definite period of time is affected largely through increase in shoot number, shoot weight playing a relatively minor role. This has actually been verified under field conditions (Barua & Dutta, 1959). Decrease in shoot weight with increasing age from tipping, however, has considerable effect on yield at different times of the year.

Despite variation in mean shoot weight and average shoot number per first order lateral, a good correlation ($r = 0.81$, $P < .01$) was observed between total number of first order laterals and bush yield at different tipping heights. In circumstances where the mean weight of a shoot and the average number of shoots per first order are not likely to vary appreciably, a count of the number of first order laterals can be expected to give an even better correlation with yield. This might prove to be useful for checking yield measurements.

Summary

This paper is a further analysis of the experiment described in an earlier paper (Barua & Wight, 1959). Mature, annually pruned tea bushes belonging to a clone were tipped at heights varying from 5 to 35 cm, rising in steps of 5 cm. The weight of a shoot decreased and the number of dormant (*banjhi*) shoots increased with the reduction of the tipping height. At all tipping heights, shoot weight and apical activity diminished with increasing age from tipping.

Each axil bud on a primary can be supposed capable of forming a branch (lateral) of the first order. The number of axil buds actually forming first order laterals was found to decrease as the distance between the bud and the tipping level increased. The uppermost axil bud on every tipped primary formed a first order lateral.

The number of first order laterals did not increase in proportion to the length of primary below tipping. On an average, four first order laterals were produced by a decapitated primary.

Every first order lateral produced, on an average, two shoots of the second order. Thereafter the number decreased gradually with increasing complexity of the branch system.

Eight orders of lateral were produced in the course of the plucking season at tipping heights of 15 cm and above, and seven orders at 5 cm and 10 cm tipping heights. The fourth order laterals yielded the maximum number of shoot at all tipping heights.

The maximum number of shoots, making 40-60 per cent of the total yield, was harvested from the uppermost first order lateral. The number of plucked shoots diminished in proportion to the distance of the lateral from the point of tipping. The fifth and lower first order laterals, which were very few, made an insignificant contribution towards yield.

The number of shoots harvested from a vigorous primary increased steadily until the end of the plucking season, while the number of shoots plucked from a bush as a whole diminished rapidly from early October. This suggests that the non-growing buds on comparatively weak shoots out-numbered at an increasing rate the growing buds on the vigorous shoots.

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EXPERIMENTAL STUDIES ON FEMALE REPRODUCTIVE STRUCTURES OF *CITRUS MICROCARPA* BUNGE*

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Introduction

Some species of *Citrus, Mangifera* and *Syzygium* exhibit polyembryony, a phenomenon of considerable interest in morphogenesis. Culturing entire ovaries and their components excised from such plants should prove useful in interpreting the factors responsible for the formation of adventive embryos and other associated problems. Brief reports on the technique and observations on *in vitro* culture of ovules, nucelli and embryos of *Citrus microcarpa* have already been published (Ranga Swamy, 1958, 1959; Maheshwari & Ranga Swamy, 1959). This paper is a supplementary account of the data, and in addition describes the culture of fruit tissues of the same species.

Materials and Methods

Modified White's medium (WM) used in LaRue's laboratory, University of Michigan, U.S.A. (private communication) was employed as control. Its composition is as follows (in mg/l): (a) $MgSO_4.7H_2O$ — 360, $Ca(NO_3)_2.4H_2O$ — 260, Na_2SO_4 — 200, NaH_2PO_4 — 165,

KNO_3 — 80, KCl — 65; (b) $MnSO_4.4H_2O$ — 3, $ZnSO_4.7H_2O$ — 0·5, H_3BO_3 — 0·5, $CuSO_4.5H_2O$ — 0·025, Na_2MoO_4 — 0·025, $CoCl_2$ — 0·025; (c) $FeC_6O_5H_7.5H_2O$ —10; (d) glycine — 7·5, niacin — 1·25, thiamine hydrochloride — 0·25, calcium pantothenate 0·025, pyridoxine hydrochloride 0·025; (e) indoleacetic acid — 1; and (f) sucrose — 20,000.

The media were made by dissolving all of the soluble components in double-distilled water in which Difco Bacto-agar (0·8 per cent) was suspended, distributing to culture tubes, plugging and autoclaving at 15 lb for 20 minutes. Ovaries and ovules were obtained from locally grown plants.

Observations

OVULES — Ovaries, and ovules excised from unpollinated pistils did not prove amenable to culture. Ovules (post-fertilized) in which nucellar embryos had been initiated responded to the treatments (see Ranga Swamy, 1959). They developed callus masses which were of a friable nature and burst through the outer integument, mainly at its two ends and on the raphe.

Two regions were seen in them: (i) a narrow, neck-like portion at the rupture of the integument and (ii) a peltate, head-like zone spreading out from it (Fig. 1). When such masses attained sufficient size, they were subcultured but did not grow further.

The growth pattern of the embryos *in situ* was much affected. In ovules which had only the zygotic embryo, nucellar embryos originated in two weeks but their growth was not sustained beyond the heart-shaped stage. However, in ovules cultured just after the initiation of nucellar proembryos, the latter developed normally on WM modified to contain 5 per cent sucrose. On WM+2, 4-D (5 ppm) calluses were produced instead of embryos (Fig. 2). WM+coconut milk and WM+malt extract were detrimental to the growth of embryos; no new embryos were ever initiated, and even those already present failed to grow.

On most media the ovules looked like mature seeds, and in some cultures maintained beyond two weeks the seeds ruptured in the micropylar part and the embryos pushed forth (Fig. 3).

NUCELLI — Young nucelli having only one or two proembryos, showed beginnings of necrosis in less than two weeks. The tissue began shrinking from the chalazal end followed by a browning until the whole of the nucellus shrivelled.

With entire nucelli also (containing torpedo-shaped embryos) the results were not encouraging. The nucelli remained healthy for two weeks, but never proliferated either on WM or WM+casein hydrolysate (400 ppm). The embryos *in situ* showed a little development, but eventually died. On WM+gibberellic acid (2 ppm) and on the same medium supplemented with casein hydrolysate (400 ppm) or kinetin (0·02 ppm), the basal portion of the nucellus swelled into a big hump, the micropylar part was completely obliterated and the embryos within were exposed. In about two months, the embryos grew into long, wiry seedlings and their cotyledons became massive or at times cylindrical, simulating lateral branches of an axis (Fig. 4). Roots were not formed and the embryos were still enclosed by the dead nucellar tissue; on excision, the embryos showed a little callus on their radicles.

When the chalazal halves of young nucelli were cultured, they turned brown and flaccid overnight. No growth occurred either on WM or the same supplemented with growth substances like casein hydrolysate, coconut milk, tomato juice, malt or yeast extract.

The micropylar halves of nucelli[1] were also cultured. On WM the nucelli bearing torpedo-shaped embryos swelled. Either the cotyledons or the radicle of the embryos pushed forth and remained healthy for two months, but the nucellar tissue turned brown and papery.

The control medium (WM) alone was insufficient to sustain the growth of young nucelli. On fortifying it with casein hydrolysate (400 ppm) the results were encouraging, although in the first two or three weeks of culture the nucelli did not show appreciable growth. Later pearly, white, sometimes green, tumoroid outgrowths developed abundantly over the entire surface of the nucellus (Fig. 5). These have been termed " pseudobulbils ". By the sixth week they formed a fragile tissue which had increased to 8-10 times the volume of the original piece, and the proliferation was so extensive that neither the limits of the original tissue nor the proembryos could be recognized. As growth proceeded, an unlimited number of pseudobulbils was produced. Nucelli cultured on WM for some time and then transferred to the same medium supplemented with casein hydrolysate also produced pseudobulbils. Dissections of ovules of the same age (10-12 weeks old) in nature showed several differentiated embryos although most of the nucellar tissue had already been consumed.

Squashes and microtome sections revealed that the young pseudobulbils were uniformly parenchymatous (Fig. 6). Either the entire mass or only the core of the pseudobulbil was meristematic. Divisions in random directions produced pseudobulbils of bizarre forms (Fig. 7).

Proliferation of the pseudobulbils also occurred as the result of a superficially

1. Unless otherwise mentioned, hereafter the term nucellus implies the tissue from the micropylar portion of the ovule.
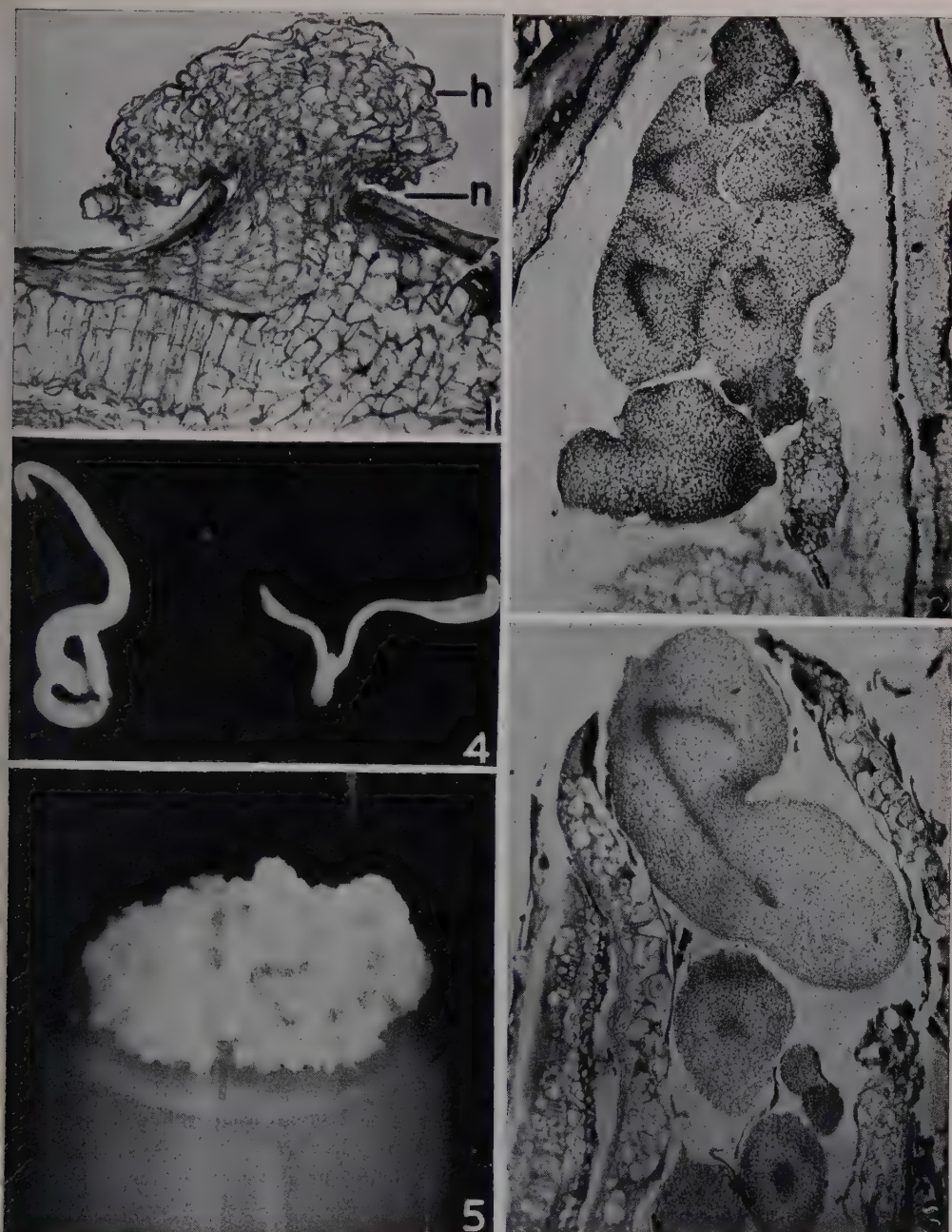
FIGS. 1-5 — (*h,* head region; *n,* neck region). Fig. 1. L.s. integumentary overgrowth. × 159.   Fig. 2. L.s. micropylar portion of 3 days old ovule on WM + 2, 4-D (5 ppm); note the atypical embryos. × 58.   Fig. 3. L.s. micropylar portion of 2 weeks old ovule on WM + sucrose (3 per cent); observe the obliteration of the tissue at the micropyle and the emergence of the radicle of the uppermost embryo. × 56.   Fig. 4. One month old seedlings excised from a nucellus cultured on WM + GA (1 ppm) + casein hydrolysate (400 ppm). × 5.   Fig. 5. A culture of nucellus showing formation of pseudobulbils. × 1·9.
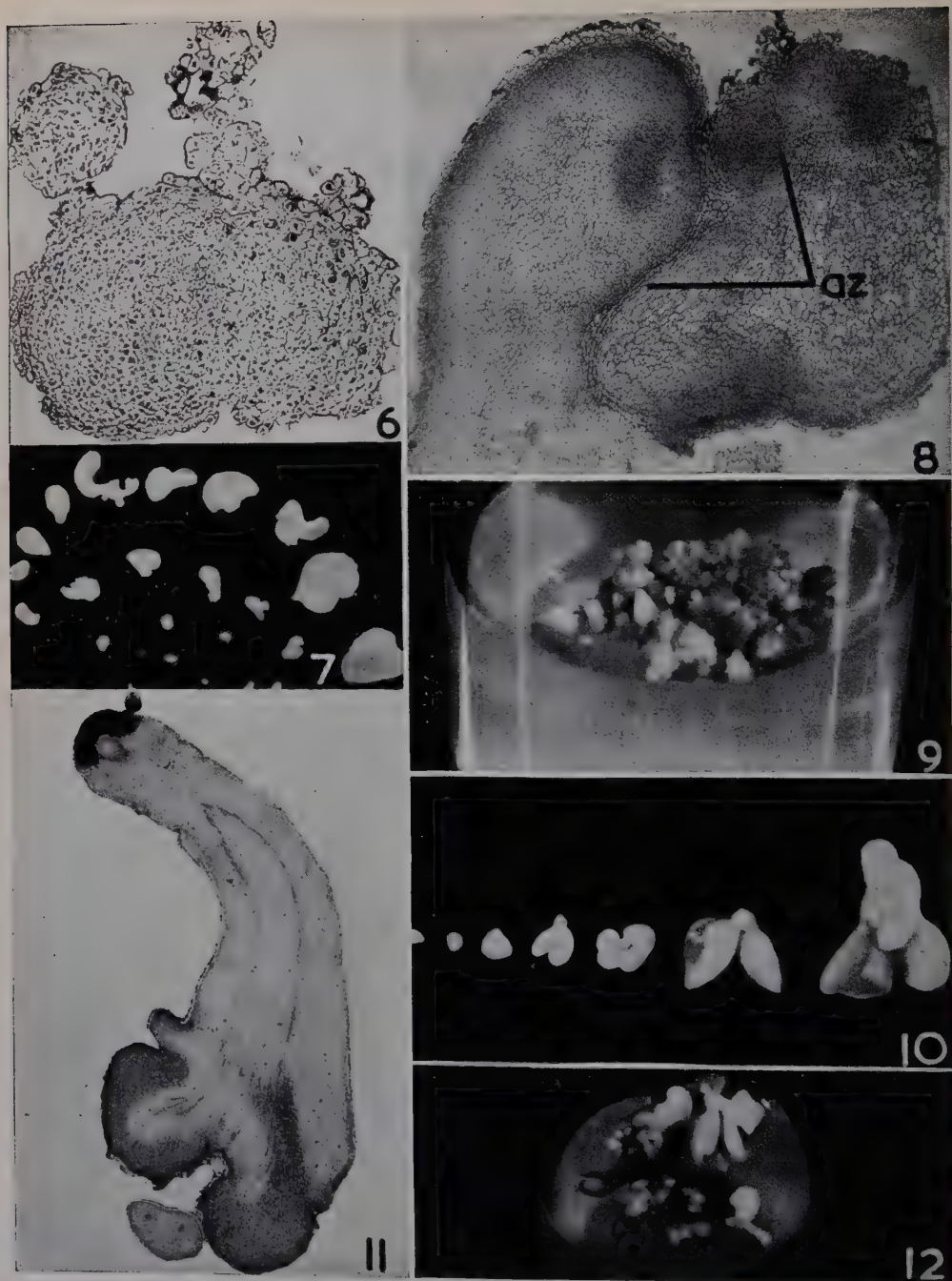
FIGS. 6-12 — (*az*, zone of separation). Fig. 6. Section through a young pseudobulbil. × 125. Fig. 7. Pseudobulbils of different shapes and sizes. × 1·5. Fig. 8. Section through a mass of pseudobulbils showing zones of separation. × 56. Fig. 9. Culture showing pseudobulbils in early stages of differentiation into embryos. × 2·3. Fig. 10. Globular and fully developed embryos isolated from cultures of pseudobulbils. × 4·5. Fig. 11. L.s. embryo differentiated from pseudobulbil. Provascular strands and callusing of root and cotyledons are seen. × 95. Fig. 12. Embryos of diverse forms in a culture of pseudobulbils. × 1·2.

organized meristem which gave rise to periodic growths of new tissue. Pieces of this tissue severed from the main mass due to the formation of individual epidermal layers around them (Fig. 8), and renewed their activity.

Frequently, the pseudobulbils became transformed into embryos (Figs. 9, 10). Provascular strands differentiated in them (Fig. 11). Hypodermal lysigenous oil glands, similar to those in embryos developing in nature, were also observed. The cotyledons exhibited diverse forms (Fig. 12) and tended to be massive, coarse and green. Pluricotyly was common.

The embryos developed from pseudobulbils often differentiated into seedlings which presented abnormalities of cotyledons, stem and hypocotyl. However, the leaves were quite normal like those of the mother plant (Fig. 13). The older leaves abscised as new ones developed. Occasionally, seedlings also produced calluses recalling pseudobulbils. In several instances these were seen developing from the hypocotyl region, and rarely from the root portion.

When quiescent, the pseudobulbils appeared coralloid and spongy, but gradually turned brown. The outermost layers appeared corky and the hypodermal cells were packed with starch grains (Fig. 14). The cells in the interior were large and showed thickenings at the corners. Tracheids were found interspersed in the tissue (Fig. 15). Sometimes, the pseudobulbils developed into 'woody' structures (Fig. 16).

Subcultures of Pseudobulbils — Portions of pseudobulbils were subcultured on basic medium (WM) alone or supplemented with one or more growth substances like adenine, casein hydrolysate, gibberellic acid and kinetin. Single pseudobulbils merely enlarged, some considerably. Only when a small mass of tissue (about 17-20 mg) was subcultured, there was resumption of growth of the inoculum. However, the pseudobulbils did not survive on WM.

In liquid " shake " cultures made with WM+adenine (20 or 40 ppm) the pseudobulbils showed unlimited growth and proliferation. When the much enlarged pseudobulbils were transferred to fresh medium, they tended to develop into embryos. However, no normal seedlings

were formed. 40 ppm adenine proved more effective than 20 ppm in eliciting the enumerated responses.

When an agar medium (WM+adenine) was used, growth was inhibited. Even in five months old cultures the pseudobulbils neither proliferated nor differentiated.

Addition of gibberellic acid (1 ppm) to WM did not favour proliferation but promoted root formation in pseudobulbils. If both kinetin (2 ppm) and casein hydrolysate (400 ppm) were added to the control medium, the rate of proliferation of pseudobulbils was accelerated. Subsequently, however, the growth was as good as that on a medium without kinetin. In a lower concentration of kinetin (0·02 ppm)+casein hydrolysate (400 ppm) no appreciable change was noticed. The several abnormalities manifested on WM+ casein hydrolysate also appeared on media containing kinetin.

Mostly 400 ppm of casein hydrolysate were used. Concentrations of 100, 200 and 600 ppm were also tried, but best growth of pseudobulbils was obtained on WM+casein hydrolysate — 400 ppm (pH 5·8 before autoclaving). The pseudobulbils have now been maintained for four years through repeated subcultures.

EMBRYOS — To start with, fully formed but immature embryos were cultured to determine a suitable medium for their growth (*see* Maheshwari & Ranga Swamy, 1959). Later younger embryos (Fig. 17) and nucellar proembryos (Fig. 18) were reared.

Fully Formed but Immature Embryos — On modified White's medium devoid of sucrose, one week old embryos showed primary root (about 2 mm long) and copious root hairs, but lateral roots were formed only rarely. In three weeks the two primary leaves completely unfolded and the hypocotyl elongated carrying with it the upper cotyledon. Both the cotyledons were, however, abscised sooner or later. The seedlings were feeble and soon turned yellow.

On WM (with 2 per cent sucrose) the embryos rooted within a week. The primary root showed a 50 per cent increase in length over that from embryos grown on WM without sucrose. By the eleventh
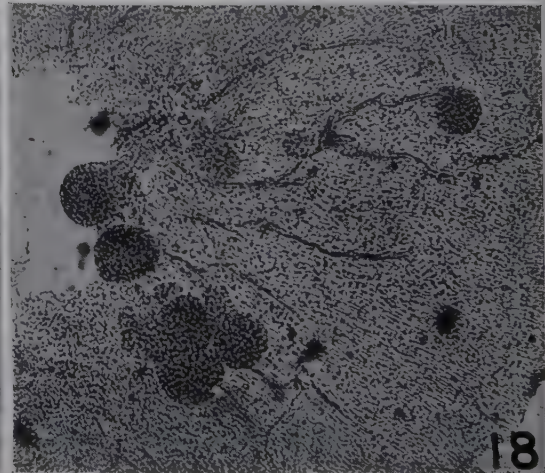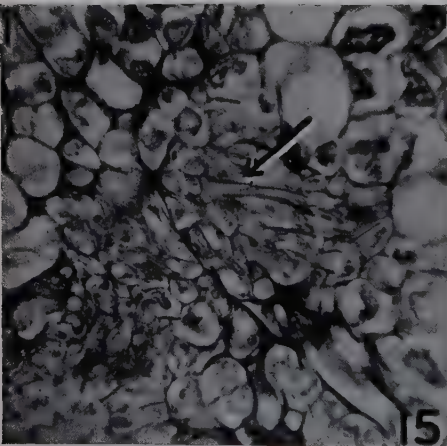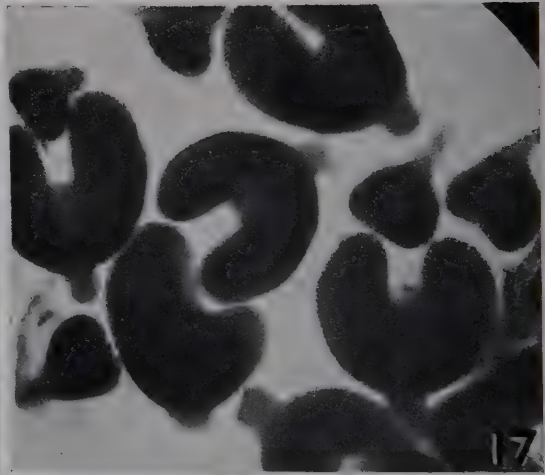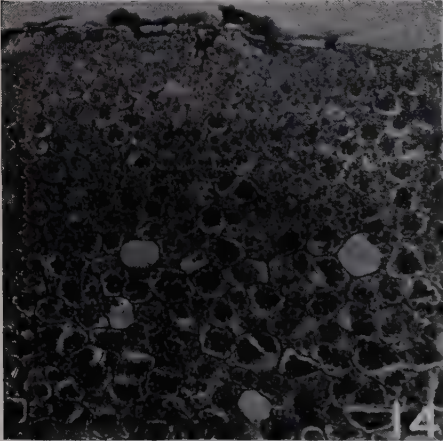
day the embryos had swollen hypocotyls and long tap-roots. Unlike the condition on WM without sucrose, here both root and shoot growth was favoured. The seedlings were stouter and more vigorous than those obtained on the non-sucrose medium.

Tukey's medium proved equally satisfactory for the growth of seedlings. Lateral roots originated earlier on Tukey's medium containing 4 per cent sucrose and 1 ml/l trace elements solution than on any other medium.

In seedlings kept growing for two months on WM+IAA (5 ppm), the roots coiled at the bottom of the culture vial probably due to lack of free space. Nodule-like structures were formed at random on these roots. On WM+malt extract (0·5 per cent) the seedlings were stunted, but on WM+yeast extract (0·5 per cent) their growth was better. In embryos grown on WM+gibberellic acid (1 ppm)+ casein hydrolysate (400 ppm)+kinetin (0·01 ppm) both the stem axis and leaves became elongated and wiry; the cotyledons were somewhat massive, the hypocotyl tip showed a slight callusing and did not develop into a normal root.

On all the above media the growth of the seedlings was favoured in dark, at least in early stages, but prolonged darkness caused etiolation. On WM+casein hydrolysate (400 ppm) only weak seedlings were produced even in dark (Fig. 19).

An abnormal behaviour of the embryos was observed when 5 or 10 ppm 2, 4–D were added to WM, probably due to the high concentrations of the substance (Fig. 20). Invariably, the surface of the cotyledons away from the medium became coarse accompanied by loss of green colour and formation of groups of meristematic cells (Fig. 21). Concomitantly, the surface in contact with the medium also callused and became ruptured exposing

the underlying mesophyll. So frail was this callus that it easily separated into individual cells when cultured in a liquid medium put on a shaker. Portions of this inoculum were subcultured, but they did not grow. Normal seedlings (obtained on WM) also succumbed when transferred to WM to which 2,4–D had been added. First the hypocotyl and subsequently the root and the cotyledons became disrupted resulting in necrosis of the tissues.

The seedlings raised on WM+NOA (5 ppm) developed no leaves even after three months in culture, but were otherwise normal. When they had reached a length of 25 mm, their stem tips (2 mm from the apex) dried up and the immediate subjacent portion (about 4 mm) became top-shaped. Soon some buds originated from this region. These developed as marginal protuberances, were green and showed a group of closely packed meristematic cells (Fig. 22). Sometimes the buds arose from the inner layers of the stem (Figs. 23, 24).

Since the above experiments showed that modified White's medium (WM) sustains normal growth of differentiated but immature embryos, it was used as control in subsequent work on younger embryos.

Torpedo-shaped Embryos — Embryos of torpedo shape inoculated on WM swelled initially but turned brownish yellow within three days of culture. Rarely the radicle showed some intumescences which became profuse when the concentration of sucrose was raised to 3 per cent. The growth, however, was not normal. The cotyledons showed swellings and intumescences. The meristematic cells of the stem apex became inactive, showed vacuolation and loss of contents.

On the control medium modified to contain 5 per cent sucrose, the embryos

←—

Figs. 13-18 — Fig. 13. Growth of seedlings from pseudobulbils. Leaves are normal. × 2·8. Fig. 14. Section of 3 months old pseudobulbil; the cells are full of starch grains. × 119. Fig. 15. Same as Fig. 14; showing a tracheid in the mass (arrow-marked). × 130. Fig. 16. A bizarre ' woody ' pseudobulbil from an old culture. × 3·7. Fig. 17. Embryos dissected from a young ovule (*in vivo*). × 12. Fig. 18. Whole mount of nucellar tissue showing proembryos (*in vivo*). × 7.
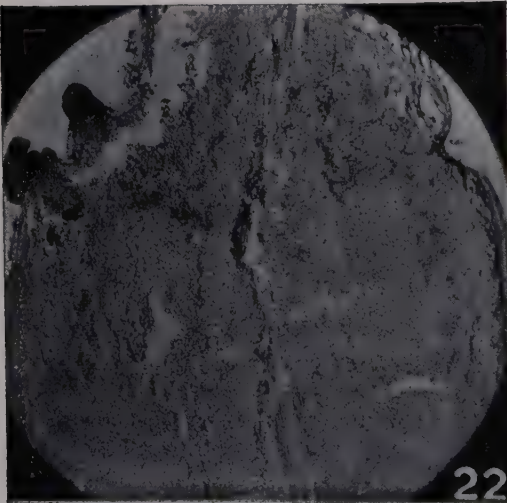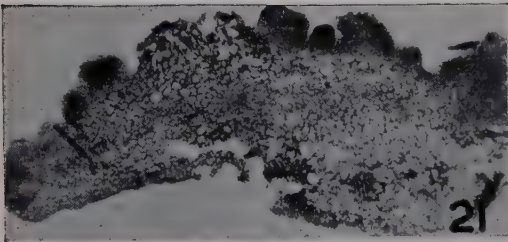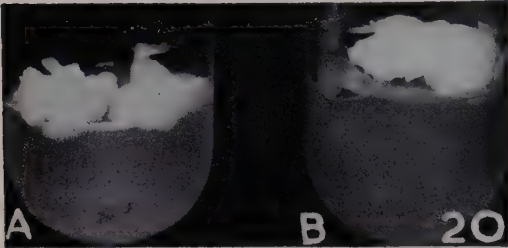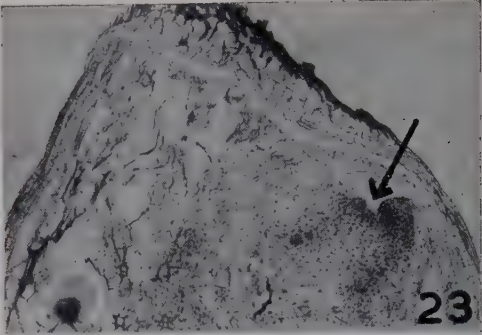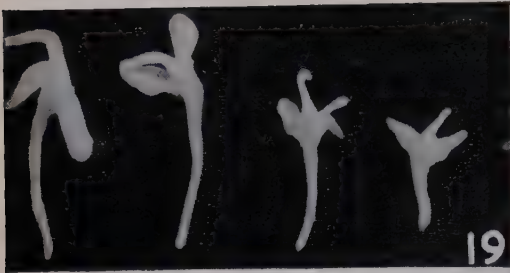
grew into weak seedlings. The retention of the maternal tissue, namely the nucellus, did not benefit their growth.

There was a marked response on WM+ casein hydrolysate (400 ppm). The embryos swelled considerably and appeared corky. The cotyledons were unequal in size and in the majority one or both the cotyledons became bilobed. A blunt protrusion developed in place of the plumule making the embryos appear tricotyledonous. The protuberance remained green and healthy but failed to develop further. In a few cultures it appeared 2-lipped. All these changes covered a period of 8-13 weeks. When transferred to WM+kinetin (0·01 ppm) such embryos developed a short root, rather precociously, but the green papilla remained dormant.

Older torpedo-shaped embryos cultured on WM+coconut milk (40 per cent) swelled considerably; their cotyledons became funnel-like but were rather stunted, and the plumule developed into a short axis bearing leaf primordia (Fig. 25). Younger embryos behaved somewhat differently. Their hypocotyls showed a slight swelling while the cotyledons were elongated. The growth of the plumule was suppressed and the growing point necrosed (Fig. 26). The primary root was not formed either in the young or old embryos.

In embryos reared on WM+thiourea (20 ppm) the stem tip did not develop; the internal cells were large and vacuolate and the peripheral cells appeared suberized (Fig. 27). The radicle showed a tendency for callusing. The cotyledons were rigid, massive and green even after two months in culture. One of them always outgrew the other.

Barbitone (5 ppm), which is reported to be a carcinogen, was also used. When added to WM, its effects were in many respects similar to those obtained on modified White's medium containing 3 per cent sucrose. The growth was not normal, the plumule projected like a papilla and the cotyledons curved backward and sometimes formed a pouch-like structure at their tips.

Heart-shaped Embryos — Those cultured on WM enlarged extensively, turned yellow and died in three days. When reared on modified White's medium containing 3 per cent sucrose the embryos generally enlarged and the two lobes developed into cotyledons.

All the embryos cultured on WM+ casein hydrolysate (400 ppm) matured in 14-18 days. Many showed pluricotyly, the tetracotyledonous embryos appearing like twin embryos (Fig. 28).

Globular Embryos — On the control medium (WM), the fate of isodiametric (0·014-0·028 mm along both the axes) proembryos was similar to that of embryos of other ages. No growth was obtained beyond a little swelling and they succumbed in three days. Figure 29 shows a proembryo 27 days old in culture.

There was no appreciable change in the growth of proembryos when the concentration of sucrose was raised to 3, 5 or even 10 per cent. However, they remained healthy for as long as 20 days on media containing 5 or 10 per cent sucrose unlike those on media with 2 or 3 per cent sucrose. With 5 per cent sucrose the epidermal cells of the suspensor showed short projections and the breadth of the suspensor almost equalled that of the apical portion of the embryo, while on 10 per cent sucrose

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FIGS. 19-25 — (*lp*, leaf primordium). Fig. 19. Seedlings from 2 months old cultures on WM + casein hydrolysate (400 ppm). × 4. Fig. 20. Three weeks old cultures of immature embryos on WM + 2, 4-D (A, 5 ppm; B, 10 ppm) showing callused cotyledons. × 1·3. Fig. 21. L.s. cotyledon of embryo callused on WM + 2, 4-D (10 ppm) showing groups of meristem and ruptures on upper and lower surfaces respectively. × 50. Fig. 22. L.s. portion of stem (subjacent to the apex) from a seedling reared on WM + NOA (5 ppm); 2 buds are seen, the lower of which has differentiated. × 34. Figs. 23, 24. Same as in Fig. 22; in Fig. 23 one incipient bud (arrow-marked) and in Fig. 24 two endogenous buds and the discontinuous outer tissue are seen. Fig. 23. × 55, Fig. 24. × 57. Fig. 25. L.s. 9 days old embryo grown on WM + coconut milk (40 per cent); the plumule is swollen, elongated and bears leaf primordia. × 67.
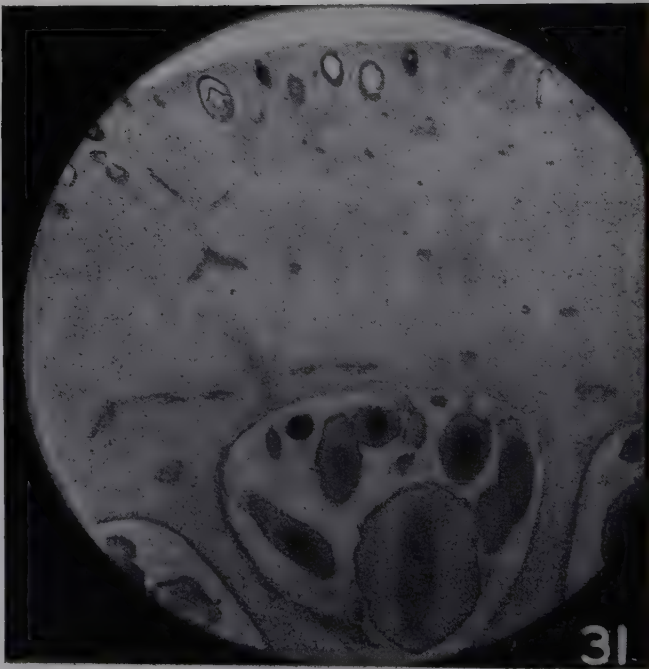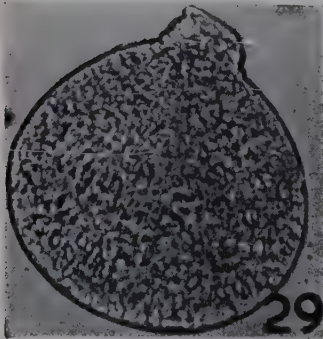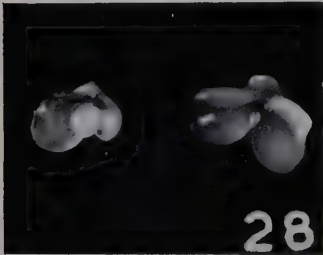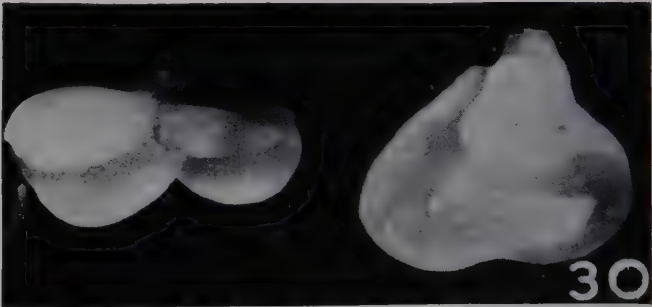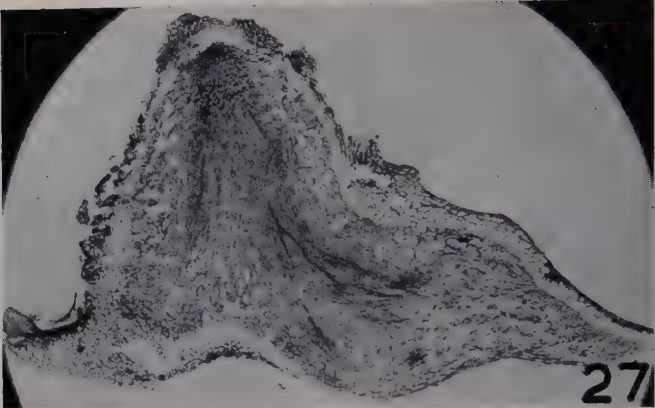
the embryos enlarged to twice their original size.

Coconut milk (25 per cent) did not prove superior to the control medium. To start with, the embryos showed some enlargement but remained quiescent even when left for a whole month; finally they turned brownish black.

Proembryos cultured on WM+casein hydrolysate (400 ppm) did much better. The cotyledons were initiated in the form of lobes and the embryos continued their pregerminal development. Growth proceeded normally through the usual stages: proembryo → heart-shaped → torpedo-shaped → mature embryo (Fig. 30). In 3-4 weeks fully organized embryos were formed in 80 per cent of the cultures.

However, embryos reared *in vitro* showed deviations from those developed *in vivo*. In nature they are deep-green, possess two massive cotyledons and a well developed radicle. On the contrary, the embryos obtained in cultures were glossy- or ivory-green, and possessed broad, thin cotyledons. Pluricotyly was frequent. One or more cotyledons showed lobations indicating syncotyly. The radicle end was thick and massive.

In one week from the time of differentiation the embryos grew into seedlings which were comparable in most respects to those obtained from mature embryos, but less robust.

FRUIT TISSUES — Tissues from 1-1½ months old fruits (Fig. 31) were cultured on media containing growth substances like adenine, coconut milk, gibberellic acid, indoleacetic acid and malt extract. Similar responses of the various tissues occurred on all the media.

Fruit Explants — Young and immature fruits with the pericarp not yet fully differentiated were sliced into four equal parts which were implanted on the medium. In about a month meristematic activity was seen in cut surfaces, the pericarp and the axile placentae (Figs. 32-34). Knobs of tissue in the form of macroscopic nodules were formed, indicating a lack of uniformity in growth (Fig. 35). These originated from the peripheral cells of the tissue explants. If they developed endogenously, the marginal layers of the fruit slices sloughed off and exposed the inner tissue. In later stages the explant presented a callus-like appearance. The nodules were friable and abscised from the parent tissue after which they either proliferated further or remained as cell aggregates. Radial rows of cells were observed in regions where proliferation occurred (Fig. 36). These effects were pronounced on WM+IAA (5 ppm) and WM+coconut milk (50 per cent).

The ovules and juice vesicles attached to the fruit explants also showed certain changes. The outer integument developed an extensive overgrowth (Figs. 37, 38). Unlike the response of excised ovules, this growth was profuse on WM+coconut milk (50 per cent). The arrangement of the cells in the epidermal and one or two hypodermal layers of the nucellus (Fig. 37) suggested that of a meristem. Sometimes the tissues internal to the integuments presented a necrosed appearance (Fig. 38); but probably such an ovule was destined to be sterile. However, no growth of the endosperm or embryos could be observed even in healthy ovules.

The juice vesicles exhibited varying patterns of growth (Fig. 39). Often they were bloated in the middle portion, and at times their tips forked. Differential activity made the juice sac bend like an arc in which the cells grew out into filaments (Fig. 39). The proliferation of juice sacs was more extensive in the club-shaped apical portion than elsewhere

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FIGS. 26-31 — (*p*, plumule).   Fig. 26. L.s. 9 days old embryo grown on WM + coconut milk (40 per cent) showing the necrosed plumule. × 47.   Fig. 27. L.s. embryo reared on WM + thiourea (20 ppm) for 7 weeks; the peripheral cells appear suberized. × 34.   Fig. 28. Development of heart-shaped embryos grown on WM + casein hydrolysate (400 ppm) for 18 days. × 6. Fig. 29. Whole mount of proembryo from a 27 days old culture on WM. × 16.   Fig. 30.   Mature embryos obtained in 1 month old culture on WM + casein hydrolysate (400 ppm). × 6. Fig. 31. T.s. portion of young fruit (*in vivo*). × 34.
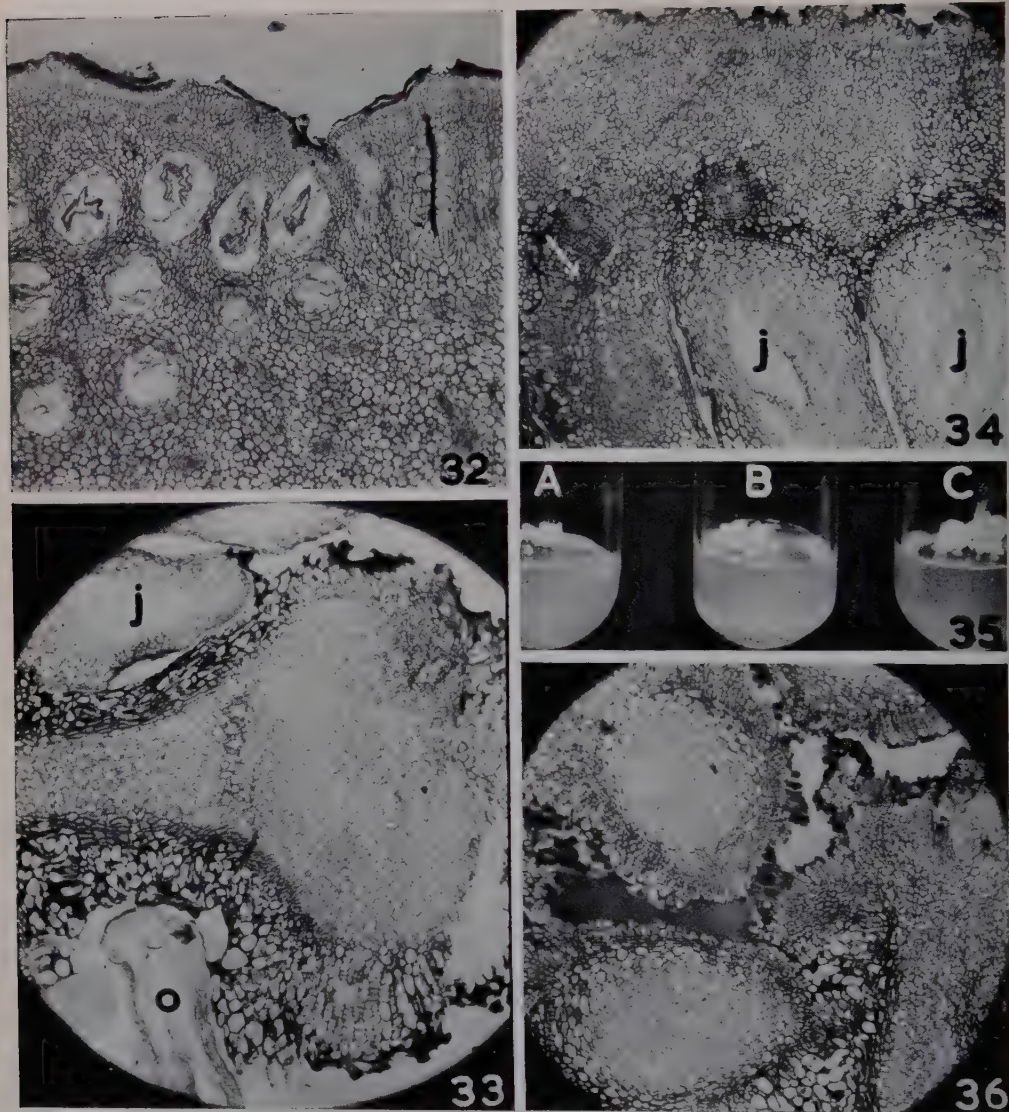
FIGS. 32-36 — (*j*, juice sac; *o*, ovule). Fig. 32. T.s. portion of pericarp reared for 1 month on WM + IAA (5 ppm) showing meristematic activity in exocarp. × 59. Fig. 33. As in Fig. 32; the placenta shows extensive proliferation; portions of juice sacs and ovule are also seen. × 40. Fig. 34. As in Fig. 32; meristematic activity is seen in the endocarp (arrow-marked); portions of 2 juice sacs are also seen. × 40. Fig. 35. One month old cultures of fruit explants; A, WM + adenine (40 ppm); B, WM + adenine (40 ppm) + IAA (5 ppm); C, WM + IAA (5 ppm). × 0·9. Fig. 36. T.s. portion of tissue from C in Fig. 35. × 32.

(Fig. 40) and occurred more vigorously on WM+malt extract (0·5 per cent) than on WM alone.

Isolated Juice Vesicles — At culture the juice vesicles (excised from 1-1½ months old fruits) were multicellular parenchymatous structures sharply distinguishable into a basal stalk and an apical club-shaped portion. Their responses were similar to those shown by intact juice sacs in cultures

of fruit slices. On WM proliferation was scanty and confined to the stalk portion. On WM+GA (2 ppm) intumescences appeared from the surface at random and in three weeks proliferation became extensive and root-like processes developed, although rare (Figs. 41, 42). As growth advanced they turned yellow and the epidermal layer was ruptured at places exposing the internal mass of glistening cells.

Discussion

In recent years tissue culture techniques have proved valuable in probing into the twin problems of growth and nutrition of excised plant tissues and organs. In the following pages the progress achieved in culturing reproductive organs, like embryos, ovules and nucelli, and fruit tissues, especially of *Citrus*, has been discussed in the light of contemporary data.

Mature embryos are known to grow on simple nutrient medium. Recently Ohta & Furusato (1957) have found that embryos of *Citrus natsudaidai* grow better en a non-sucrose medium than on a sucrose containing medium. My observations on *C. microcarpa* indicate that although the growth of immature embryos is better on a non-sucrose medium in the initial stages, the seedlings obtained are weaker than those secured on a sucrose-fed medium.

By adding barbiturates to the medium, Curtis (1947) procured tumoroid growth on orchid embryos. Subcultures of such calluses exhibited as many as seven patterns of growth (Curtis & Nichol, 1948). Seedlings were obtained from some of them. In *C. microcarpa*, however, barbitone did not prove carcinogenic beyond inducing slight intumescences on the embryos. Instead, the peripheral layers of cells, particularly those of the radicle, became necrosed arresting the growth of embryos.

Embryos (of all ages) of *C. microcarpa* eadily callused in media containing 2,4-D. Single cells were easily obtained when these callus masses were grown in a liquid medium put on a mechanical shaker. 2,4-D acted as a carcinogen with rye embryos also (Carew & Schwarting, 1958). The rye embryo callus showed capacities

for continued growth which was favoured best by the addition of casein hydrolysate.

Experimenting with a number of selfed and hybrid embryos (torpedo stage) of *Datura*, Sanders (1950) observed a spreading of the cotyledons and premature production of roots and plumules. " Multiple growth " was also noticed in embryos cultured on a modified Tukey's medium containing malt extract (0-5 per cent). Multiple growth consisted of structures showing many embryo-like outgrowths sometimes resembling normal embryonic stages. They were either independent of one another or joined in a type of fasciated growth. Similar results were obtained with *C. microcarpa* on the control medium (WM)+coconut milk (30 per cent) although rare. However, advanced torpedo stage embryos cultured on WM exhibited no such features.

While the problems relating to the *in vitro* culture of mature embryos are less complicated, the artificial culture of very young embryos is still uncertain. The young embryos are difficult to excise, liable to be mutilated or otherwise injured, and no standard medium is known for sustaining their growth. Investigations on such embryos have, therefore, been a few (*see* Rappaport, 1954).

Embryos in the process of development have been observed to delete embryonic growth in culture media and germinate precociously to produce small and weak seedlings (LaRue, 1936). Van Overbeek *et al.* (1941) were the first to report the existence of " embryo factors " like those in coconut milk which promoted the growth of young embryos. They found that unautoclaved coconut milk supported normal growth of *Datura* embryos. Since then alcoholic extracts of almond meal, banana, *Datura* ovules, non-dormant apple buds, wheat germ, peat and yeast extracts, sodium nucleate, tomato juice and casein hydrolysate have been reported to possess embryo factors.

Lofland (1950) observed that a high percentage of cotton embryos grew in low concentrations of casein hydrolysate. Ziebur *et al.* (1950) reported that, besides being an embryo factor, casein hydrolysate served as a nutrient for young barley embryos. They considered that while the amino

acid and sodium chloride components of
the commercial acid-hydrolysed (vitamin-
free) casein hydrolysate together precluded
the precocious germination of embryos, the
amino acids and the phosphate complex
served as nutrients in promoting the nor-
mal growth of embryos. Amino acids
used alone failed to duplicate the effect of
casein hydrolysate. Ziebur *et al.* (1950),
therefore, concluded that the growth of
immature barley embryos *in vitro* obtained
with casein hydrolysate " may be the
result of an interplay of both nutritional
and physical factors ". The works of
Rijven (1952, on *Capsella*) and Mauney
(1958, on *Gossypium*) on heart-shaped em-
bryos also showed that casein hydrolysate
strongly supports the growth of embryos.

Present investigations on the culture of
proembryos of *Citrus microcarpa* have
confirmed the embryo factor activity of
casein hydrolysate. Almost 100 per cent
of the proembryos cultured on this medium
grew as well as those of *Datura* on a
medium containing coconut milk (van
Overbeek *et al.*, 1941). To my knowledge
it is only rarely that such young pro-
embryos have been excised and reared
in vitro to normal seedlings with such a
high percentage of success. Neither modi-
fied White's medium (WM) alone nor the
same with the addition of coconut milk as
an adjuvant proved sufficient for their
growth. My observations differ from
those of Stevenson (1956) who reported
the beneficial role of coconut milk in em-
bryo cultures of *Citrus* spp. Only on sup-
plementing the control medium (WM)
with casein hydrolysate (400 ppm) did
the proembryos of *C. microcarpa* complete
the normal course of development and
germinate into seedlings. This suggests
that while successful growth of young
embryos *in vitro* depends upon the presence
of certain embryo factor(s), the latter may
be specific for particular species.

Although researches during the last two
decades have indicated the importance of
casein hydrolysate, coconut milk and other
substances to be of direct nutritional value
in embryo growth, there is a paucity of
more specific data on the factors controlling
the growth and development of pro-
embryos. Nor do we have adequate
knowledge of the role of nucellus and other

maternal tissues of the ovule in the deve-
lopment of embryos. Solutions to these
problems may have to be sought in the
field of ovule culture.

With the exception of a few reports,
ovule culture is a new field (*see* Ranga
Swamy, 1959). Recently Poddubnaya-
Arnoldi (1959) using the ovule culture
technique studied embryogenesis in some
orchids. Similarly, Kapoor (1959) obtain-
ed complete growth of ovules of *Zephyran-
thes*, starting from the zygote and primary
endosperm nucleus, on a defined medium.

In cultures of ovules of *Citrus microcarpa*
the growth and development of nucellar
embryos occurred normally even on the
control medium (WM) modified to con-
tain 5 per cent sucrose. On the contrary,
embryos dissected from the confines of the
ovule attained normal growth only on
addition of casein hydrolysate to the
control medium (WM). Obviously, the
more of the parental tissue that is retained,
the simpler is the nutrient medium required
and better is the growth of the organ cul-
tured[2].

While the retention of the parental
tissue may be useful in difficult cases, it is
advisable also to experiment upon the ex-
cised tissue or organ. In finding out the
factors responsible for nucellar poly-
embryony, it would be, therefore, appro-
priate to deal with the nucellus which is
the source of the adventive embryos.

Freed from the restraining influence of
the integuments and grown on a suitable
nutrient medium, the nucellar tissue of
Citrus microcarpa was activated to un-
limited growth. On transfer to fresh
medium the resulting callus masses started
new growth. These were designated
" pseudobulbils ". These nucellar calluses
perpetuated themselves and could develop
into plantlets.

Since the pseudobulbils of *Citrus* were
nucellar in origin, each one of them was

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2. This fact is also borne out by the work of
Rédei & Rédei (1955) on wheat. To circum-
vent the difficulties of excision, infection and
nutrition which the proembryos always pose,
they first cultured whole ovaries with glumes.
It is only later that the embryos (which had
progressed sufficiently in growth) were excised
from them and reared in the culture medium to
their full development. By this method they
were able to obtain seedlings from proembryos.
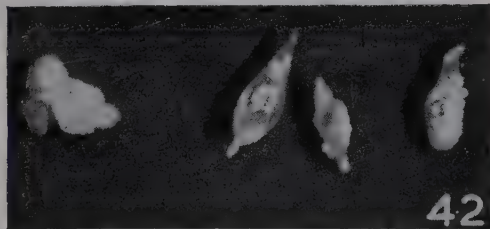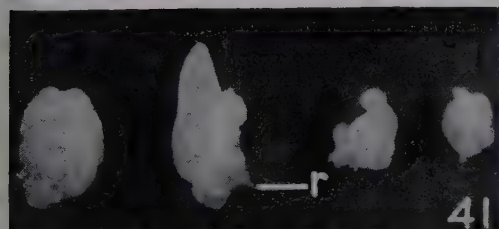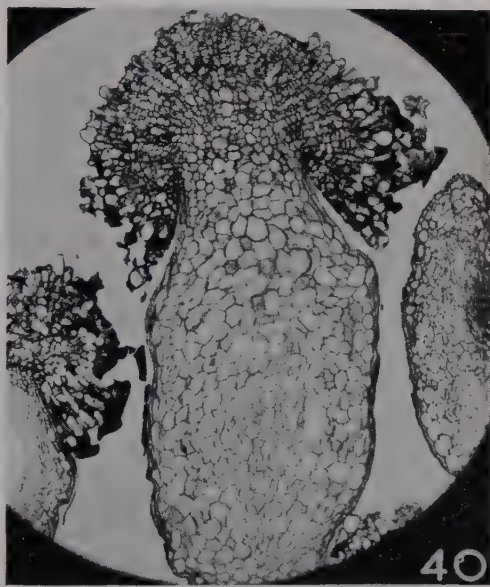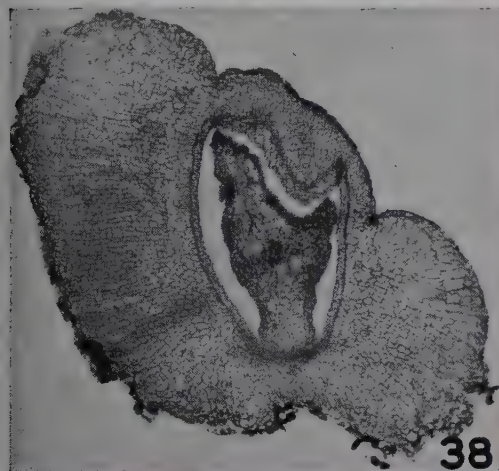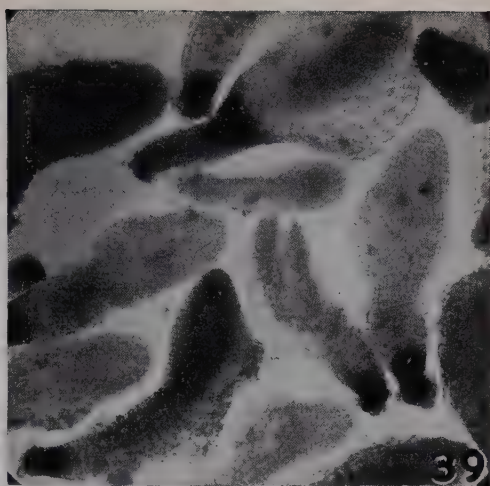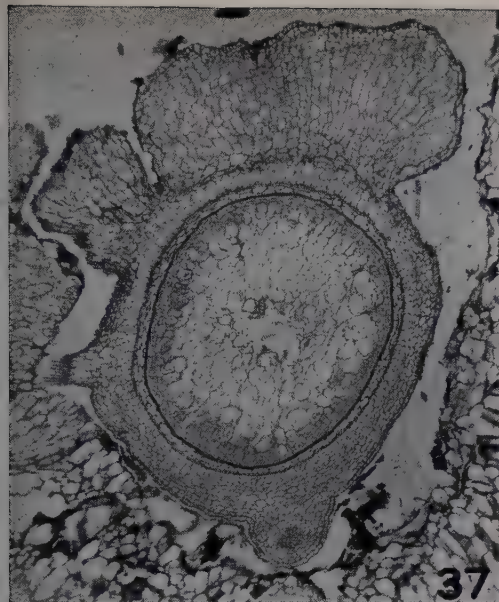
FIGS. 37-42 — (*r*, root-like process). Fig. 37. T.s. ovule from 1 month old culture of fruit explant on WM + coconut milk (50 per cent) showing extensive overgrowth of the outer integument. × 58. Fig. 38. L.s. ovule from a similar culture. The outer integument exhibits profuse hypertrophy and the tissues internal to the integuments are necrosed. × 41. Fig. 39. Whole mount of juice vesicles from a fruit explant cultured for 1 month on WM + malt extract (0.5 per cent) showing different growth patterns. × 60. Fig. 40. L.s. juice sac from a similar culture; the apical portion shows extensive proliferation. × 39. Figs. 41, 42. Juice vesicles reared for 3 weeks on WM + GA (2 ppm). All are hypertrophied. Fig. 41. × 22; Fig. 42. × 18.

equivalent to a (nucellar) proembryo, and given the suitable conditions for growth they behaved like normal embryos. Although abnormalities were seen in their root, hypocotyl or stem, the seedlings developing from pseudobulbils bore normal leaves. Nucellar cultures, therefore, indicated a method of obtaining an unlimited number of plants of the maternal parent's genotype.

It should be noted that under defined cultural conditions, an already differentiated tissue like the nucellus can be made to proliferate and then again differentiate. This completes the cycle of differentiation $\rightleftharpoons$ dedifferentiation $\rightleftharpoons$ redifferentiation in pseudobulbils (Fig. 43). Such a cycle has also been established in cultures of the carrot root-phloem cells (Steward *et al.*, 1958). Individual mature phloem cells of carrot root returned to a meristematic phase *in vitro* and proliferated into cell aggregates which on an agar medium developed into young carrot plants. Reinert (1959) has also procured embryo-like regenerates in cultures of callus tissues of carrot stem. Similarly in *Cuscuta reflexa*, Baldev (1960) has found that supernumerary embryos, differentiated from callus formed in embryo cultures, were capable of repeating the cycle.

According to LaRue (1954), " the apparently unlimited growth in culture of a tissue definitely limited in its natural growth " is of much interest. In the present work proliferation of the nucellar tissue occurred under definite nutritional conditions. Vitamin-free casein hydrolysate induced the formation of pseudobulbils. Moreover, pseudobulbils developed in cultures of nucellar tissue excised only from the micropylar region of ovules, and not in those removed from the chalazal portion. This is suggestive of a longitudinal gradient in the nucellus in regard to such activity, but what relation this bears to the influence of pollen tube and/or the zygote on the tissue surrounding the micropylar region of the embryo sac remains to be investigated.

Since it is difficult to excise young proembryos, methods of raising a crop of pseudobulbils acquire special interest. Such continually growing tissue ' banks ' would prove a potential source at all times of the year for the experimenter. Being capable of reproducing the maternal genotype and also because of being virus-free these tissue clones should prove of immense value to *Citrus* breeders. Further, with improvements in technique it should even be feasible to obtain single cells from these masses of reproductive tissue and make them behave like fertilized eggs for purposes of morphogenesis.

Investigations using the fruit tissues as isolated systems are rare. Pfeiffer (1931, 1933) and Bonner (1936) attempted culturing fruit parenchyma. Successful cultures of the soft pericarp tissues of avocado (Schroeder, 1955), citron (Schroeder & Spector, 1957) and *Ecbalium* (Nysterakis, 1960a, b) have also been reported. In citron (*Citrus medica*), gibberellic and indoleacetic acids showed a synergistic effect in inducing proliferation of the fruit tissue discs. In my experiments too, IAA caused considerable proliferation of the fruit slices.

Schroeder (1958) has experimented with several fruit species like banana, carob, feijoa, guava, lemon, macadamia, orange and papaya and states that the faculty to proliferate is common to the mature pericarp of nearly all woody, evergreen, subtropical fruit plants. Green mature olive, pumpkin and squash fruits behave similarly but comparable studies on peaches and other mature fruits of this group have all given negative results. However, Letham (1958) succeeded in obtaining a true tissue culture from explants of both young and old apple fruits prior to or following the cessation of cell divisions.

In cultures of the juice vesicles from lemon and sour orange, Stevenson (1956) secured maximum callus growth when coconut milk was added to the basic medium. Kordan (1959) has, however, reported a vigorous proliferation of mature lemon juice vesicles and the maintenance of the resulting callus culture for more than $1\frac{1}{2}$ years on a simple medium made of inorganic salts, ferric citrate and sucrose. Unlike *Citrus microcarpa*, callusing was rare in the apical portion and occurred only after primary growth had taken place in the stalk, and when the two portions were cultured separately, only the stalk proliferated.
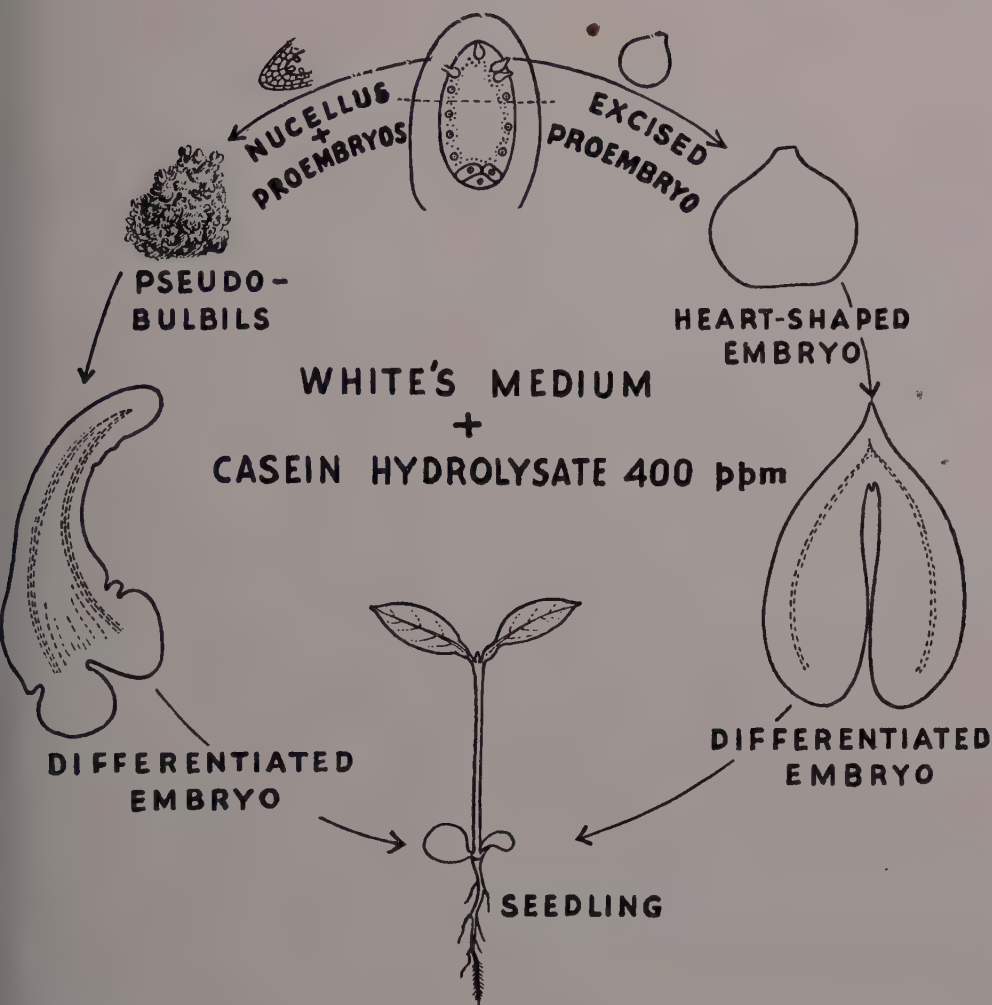
FIG. 43 — A schematic summary of the results achieved in cultures of nucelli and embryo. When the nucellus is cultured, it produces pseudobulbils some of which differentiate into seedlingss, while excised proembryos grow into seedlings without forming pseudobulbils.

One of the objectives of establishing cultures of isolated fruit tissues *in vitro* is to unravel the physiology of fruit growth. For example, it may be possible (i) to investigate the nutritional and other requirements for oil formation in the flavedo (exocarp) of citrus fruits and (ii) to understand the basis for differences in the accumulation of sugars and acids in the peel *vs.* the juice sacs of citrus fruits. Likewise, it would be of interest to inquire into the basic effects of physical factors like light on colour development in fruit tissues. In order to probe into these and other related biochemical and physiological phenomena the technique of raising perpetual cultures of fruit tissues might make possible the exclusion of factors inherent in the *in vivo* nutrition of such tissues.

Briefly then one is apt to believe in Schroeder's (1958) statement: '' that fruit tissue proliferation will behave similarly to that obtained from vegetative sources such as stem pith, hence we can

anticipate the production of highly differentiated structures such as shoot and root when proper conditions are provided."

Summary

Ovaries, ovules, nucelli and embryos of *Citrus microcarpa* were cultured under aseptic conditions in order to study their morphogenetic responses to certain growth substances and the nutritional factors controlling nucellar embryony.

Modified White's medium (WM) served as control and the cultures were maintained under laboratory conditions of light and temperature.

Ovaries (both before and after pollination) were not amenable to *in vitro* studies and yielded negative results.

Unfertilized ovules were equally unsuitable. The responses of fertilized ovules to various growth chemicals were similar so far as the integuments were concerned. The outer integument became hypertrophied at places and produced considerable masses of callus tissue. The inner integument was unaffected. Responses of the embryo were different on the several media. Normal growth and differentiation of proembryos occurred on modified White's medium containing 5 per cent sucrose; the ovules matured into seeds and even showed signs of germination *in situ*.

Nucelli excised from chalazal halves of fertilized ovules succumbed in all media. Those excised from micropylar halves of such ovules also did not grow on the control medium. However, the addition of casein hydrolysate (400 ppm) to the medium induced the proliferation of the micropylar portion of nucellus to form an indefinite number of callus masses designated " pseudobulbils ".

The pseudobulbils have been maintained for four years by continued subculturing. Some pseudobulbils developed into embryos and then to seedlings bearing normal leaves. Thus the cycle from the nucellus to embryos and then to seedlings was completed in culture.

Modified patterns of growth have been observed frequently in pseudobulbils cultured on media containing adenine, gibberellic acid or kinetin.

Nucellar embryos were cultured at different stages of development. On modified White's medium there was no growth. Medium to which casein hydrolysate (400 ppm) had been added favoured normal growth and maturity of globular embryos (0·014-0·028 mm). Later, their germination also occurred.

The inclusion of barbitone, coconut milk, gibberellic acid, thiourea or 2,4-D resulted in modified organogenesis of the embryos.

The investigations are of value to the particular problem of embryo nutrition and nucellar polyembryony, and perhaps the most useful result is the continued growth of nucelli *in vitro*.

Culture of fruit tissue explants and isolated juice vesicles also indicated the possibilities of obtaining true callus cultures from the pericarp and the juice sacs.

The results are discussed in the light of contemporary data with particular reference to the future possibilities.

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*Original not seen.

STUDIES ON THE VASCULAR COURSE
IN MAIZE PLANT

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Introduction

It is difficult to follow the precise courses of vascular bundles in monocotyledonous stems, which have numerous vascular strands scattered within the fundamental tissue. Detailed and reliable studies of this subject, therefore, had been few two decades ago when I attempted to gain a more precise understanding of vascular behavior in *Zea mays*.

I followed the vascular strands of the staminate spikelet (1940a), the axis of tassel (1939), the axillary shoot (1958a), the leaf (1940b) and the adventitious root (1958a) to their mother axis. I also studied the origin and structure of nodal anastomosis (1942) and the developmental sequence of the vertical bundles of the stem (1946). Thus the vascular strands of all the lateral organs, with the exception of the ear which was investigated during the course of my study by Laubengayer (1946, 1948, 1949), Reeves (1946, 1950) Cutler & Cutler (1948) and Lenz (1948) were followed to the mother axis. Thence, in my last paper (1958b) of the series, a general consideration of the vascular construction, with special reference to the vascular connection of lateral organs with their mother axis, was given. All these articles have been published in Japanese.

The purpose of the present paper is to summarise the results obtained and to emphasise some of my findings already reported in the Japanese articles, as well as to review the literature which has appeared during the course of my studies.

Material and Method

Vigorous plants in an open field are not suitable material for the study of vascular behavior through many nodes and inter-nodes; therefore, the varieties called Golden Bantam and Aka-shijunichi were cultivated in pots containing rather poor nutrients.

Serial microtome-sections were made through five to ten successive nodes of the stem. One plant was serially microtomed from the mesocotyl up to the tip of the tassel, but this was an exceptional case. Mature and hard materials were deminera-lized with hydrofluoric acid. Sections were stained with safranin and Delafield's haematoxylin or fast green.

In some cases, the results of anatomical studies on the vascular system were com-pared with those obtained by vital staining experiments. For this purpose a part of a living plant containing a few leaves was allowed to absorb a rosaniline solution acropetally or basipetally from a cut end. The rosaniline solution was prepared by adding the dye, which is insoluble in cold water or alcohol, to boiling water until a saturated condition was reached, and then by adding a small quantity of an organic acid such as citric or maleic. The solution moves through tracheary elements from the cut end and it stains only the walls of the tracheary elements. Permanent slides may be prepared from this material by the usual paraffin method without loss of red colour.

Discontinuous Initiation of Procambium

In the literature two patterns of pro-cambial differentiation have been reported (a) the procambium advances basipetally from a lateral organ — a leaf primordium or an axillary bud — downward toward a connection with procambial strands of the mother axis; (b) the procambium advances continuously and acropetally from the

vascular system of the mother axis upward into a lateral organ.

According to Esau (1953), the procambium differentiation of leaf traces belong to the latter pattern in most seed plants. In his studies on *Zea mays*, Sharman (1942) suggested that the main and lateral procambial strands, which are to enter a leaf primordium, are initiated near the region of leaf insertion; and that differentiation advances upward and downward.

My observations (1946) show that the median procambial strand of a leaf primordium differentiates continuously from the stem upward and toward the distal part of the primordium, although the differentiation of the lateral is the same as that described by Sharman except the seedling. Larger lateral procambial strands going to a few leaves of a seedling differentiate continuously and acropetally, as in the case of the median procambial strand.

The procambial strands which provide for an axillary bud are initiated at the base of the organ and differentiate basipetally to the mother axis (1958a). On the contrary, in the staminate spikelet, which is morphologically a kind of lateral branch, the large procambial strand of the branch trace is evidently in continuity with the vascular system of the rachis from the beginning.

The difference between procambial differentiation in a lateral branch of the vegetative axis and that of the tassel seems to depend upon the manner of development of the two. The former usually is initiated far from the shoot apex as a small primordium whereas the latter is initiated near the apex as a larger primordium. It is inferred, therefore, that the initiation of procambial strands advances acropetally from the mother axis toward the lateral organ when a large lateral primordium is initiated near the apex. The same interpretation was expressed by Wardlaw (1943) in his studies on the fern *Onoclea*. This interpretation may also be applied to procambial strands entering a single leaf primordium. Among the procambial strands of a leaf primordium, the median differentiates acropetally, while the rest develop in discontinuity with

the procambial strands of the mother axis.

Procambial discontinuity is often observed in the differentiation of the outermost peripheral vascular strands which are initiated after intercalary elongation of the stem has taken place (1946). As the tissue of the elongating zone remains more embryonic than that of other regions, the zone may fall somewhat behind the other regions in the differentiation of procambial strands. This results in the discontinuity of procambial differentiation mentioned above. At the basal part of the stem, however, intercalary elongation decreases, and the continuous pattern of procambial differentiation prevails.

Dicyclic Construction of the Vascular System

Except for the classic studies of Falkenberg (1876) and Strasburger (1891), detailed descriptions of the vascular system in maize have not been published. Therefore I (1939, 1940a, 1940b) studied the course of vascular strands from the tassel to the base of the stem in serial microtome sections. If the medullary bundles in the main axis of the tassel are followed downward, they are found to unite often with branch trace strands from many staminate spikelets and several secondary raches below. Furthermore they descend through many foliar nodes without uniting with the leaf trace strands and move gradually toward the periphery of the stem. On this basis these bundles were described as the cauline in my first paper (1939). Besides these, the medullary bundles of the leaf trace origin at the lower levels of the stem, diverge gradually toward the periphery and descend through many internodes. Although some of them unite with each other in their outward course, the region where they unite most frequently with each other is near the periphery. Such strands were called the compound bundles in my fourth paper (1942).

In Figs. 1-3, as well as in other figures of this paper, the bundles shown in white are the compound ones, most of which are arranged in an irregular circle at the sub-peripheral region. The stem bundles
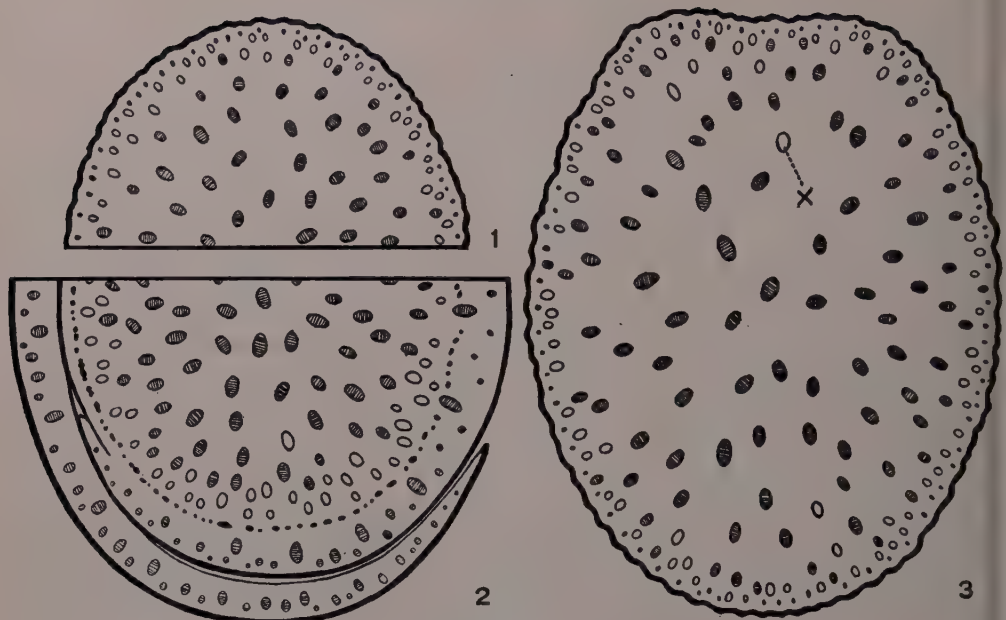
FIGS. 1-3 — T.s. through the stem of *Zea mays*. Fig. 2. Section through the sixth node, from top. Figs. 1, 3. Section through the internodes just above and below that node respectively. The minute bundles in black indicate the outer system, consisting of outermost peripheral bundles and the bundles in white the inner system, consisting of the compound bundles. The median leaf trace strand of the first (uppermost) leaf has fused with that of the second leaf during their longitudinal course. The compound bundle thus formed is labelled X in Fig. 3. The cross-hatched bundles indicate leaf trace strands which have descended without connection with any other bundles. (Kumazawa, 1940b).

shaded are the leaf trace strands derived from six leaves above and have descended without connection with any other bundles.

It was surprising to me that the compound bundles descend farther downward through the stem, staying in the sub-peripheral region, and never moving outward to connect with the outermost peripheral bundles, because the medullary bundles had been expected to come back to the outermost peripheral region of the stele in their downward course. In other words, the outermost peripheral bundles indicated in black in Figs. 1-3 and the compound bundles (including the cauline) indicated in white in Figs. 1-3 belong respectively to two quite independent vascular systems without direct connection. The two systems cannot be distinguished with certainty at a given level of an internode (Figs 1, 3), because vascular strands belonging to each come

close to one another at the periphery of the stem. In the node, however, the outermost peripheral strands themselves are arranged in a circle (Fig. 2) and can evidently be distinguished from other bundles situated at the sub-peripheral region. As will be described in a later paragraph, the two systems are respectively provided with trace strands from one and the same lateral organ.

This dual feature of the vascular system may be observed from the level of the lowermost node of the stem upward to the tip[1] of the main rachis. At the tip of the rachis the inner system originates from floral bundles and the median bundle of the lower glume, while the outer system originates from the lateral veins of the lower glume (1940a).

1. A tassel rachis terminates in one or two imperfect spikelets which usually decay and shrink in the early stage of tassel development.

Since the dicyclic construction of the vascular system was first pointed out by me in my third paper (1940b), I have paid particular attention to this feature. Sharman (1942) followed the vascular course through many nodes and internodes, but he did not point out such a feature of the vascular system. However, Laubengayer (1946, 1948), Reeves (1946, 1950) and Cutler & Cutler (1948) discovered the dual feature of the vascular system in the artificially retted cob. Laubengayer (1949) says that " the finding of the formation of two distinct vascular systems (in cob) . . . was unexpected." He (1949) also carried out a study on the vascular system in the axis of tassel, the results of which are similar in most respects to my descriptions (1939, 1940a), and he revealed a dual nature of the vascular system in the axis of the tassel somewhat similar to that of the cob. The systems were called respectively the outer system and the inner system by Laubengayer (1948) and the axial system and the peripheral system by Reeves (1950). However, nobody has ever noticed the occurrence of two distinct systems in the vegetative stem of maize.

Nodal Plexus

The horizontal network of bundles— the nodal plexus — is found at the nodes, and its histogenesis and distribution have already been described by me in detail (1942). The relationship between the nodal network and vertical bundles or trace strands from the lateral organs has been interpreted variously in *Zea* and some other grasses by de Bary (1877), van Tieghem (1884), Guillaud (1878), Strasburger (1891), Bugnon (1920a, c), Arber (1930), Sharman (1942) and others, but a common understanding has not yet been achieved.

As already stated by me (1946), the vertical procambial strands differentiate from the centre of the stem toward the periphery at a given level of the stem. The initiation of procambial strands which are to develop into the nodal network occurs in the fundamental meristematic tissue at the stage when the protoxylem elements of the vertical strands situated

at the centre of the stem have matured. In this stage most of the compound bundles in the sub-peripheral region have mature sieve elements, but all of the outermost peripheral bundles are still in the procambial stage. This results in the usual connection of the nodal network with both the sub-peripheral compound and the outermost peripheral bundles, but there is no opportunity for connection with most of the central vertical strands derived directly from the leaves above. Figure 4 shows a stage of the procambial network development, in which some vertical strands situated at the peripheral and sub-peripheral parts are connected with each other by the horizontal procambial strands.

It has been observed (1958a) that the lower ends of some branch traces become horizontal and form a part of the nodal plexus, and also it has been noted that the root trace bundles, connecting with the vertical bundles at the peripheral region of the stem, apparently elongate horizontally farther inward as a nodal network. However
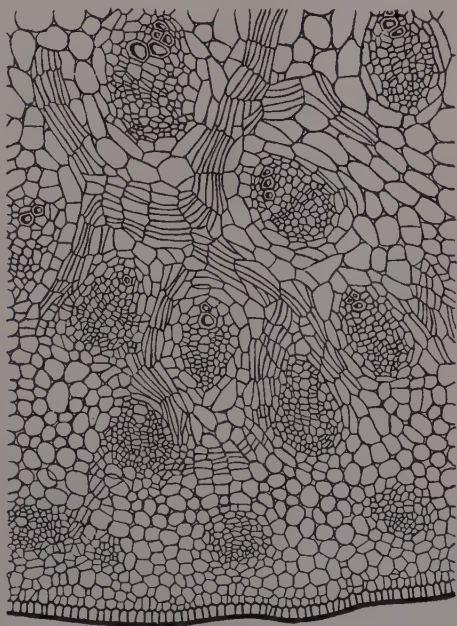

Fig. 4 — T.s. through the peripheral part of the node, showing an early developmental stage of the nodal network. × 100. (Kumazawa, 1942).

it can not be concluded, because of the lack of positive evidence, that a trace bundle which is to be supplied to a branch, a leaf, or an adventitious root does originate from the nodal plexus.

The nodal plexus develops, without exception, at a node which is accompanied by a large phyllome, such as a foliar leaf or a husk, even if the node is not accompanied by an adventitious root or a lateral branch. These facts do not suggest that the nodal plexus is a necessary feature merely for the development of the root or branch, and I am of the opinion that the establishment of a connection between the nodal plexus and the root or branch traces is rather accidental, this being due to the synchronic differentiation of the two. It is assumed that a close physiological connection may exist between the nodal plexus and the large medullary trace bundles descending from leaves above, although an actual vascular contact between the two has not been established. This assumption is emphasized by the following facts: If we allow the tracheary elements of the large leaf traces situated at the central part of the stem to absorb the acid dye solution basipetally, the dye solution diffuses out of the tracheary elements of the vertical bundles into the adjacent fundamental tissue at the node below, and this causes the tracheary elements of the horizontal anastomoses to be somewhat stained at the nodal region. The horizontal diffusion of dye solution, however, is not observed at the internodal region.

Vascular Connection between the Leaf and the Axis

Besides the classic works, leaf trace behavior of *Zea* was described by me (1940), Sharman (1942) and Esau (1943). I divided the leaf trace strands into three types: The first type (Fig. 5, M, m) is represented by the leaf trace strands which follow the inward course more or less and then move toward the periphery at the lower nodes; the second type (Figs. 5, 6, B) is represented by those which come directly to the position of outermost peripheral bundles and unite sooner or later with them; and the third type (Fig. 5, C)

is represented by those which are very minute and soon die out within the cortex at or near the level of that node.

A large leaf trace bundle is accompanied on its abaxial side by a mass of mechanical tissue which is reduced during its oblique course through the cortex of the mother axis. Within the mechanical mass a few tracheary elements, which Esau (1943) regarded as independent bundles enclosed by a common vascular sheath, are often found. My observation (1940a) reveals that the tracheary elements are differentiated only during their oblique course through the cortex of the stem and join so soon with the outermost peripheral bundles that they (Fig. 6, B_1) may be included in leaf trace strands of the second type. It is in the case of leaf traces of the first type that the vascular behavior has been discussed by many authors.

The diagram in Fig. 5 shows the various patterns of leaf trace behavior in a vegetative axis consisting of ten nodes. The leaf traces at the centre of the stem often join with other vertical strands of the same kind at or near that position before they approach the peripheral region where they usually unite. Large traces which have entered the centre of the stem do not always come back in their longitudinal downward course toward the same peripheral direction from which they entered at the node far above, but often move across the centre of the stem toward the opposite side at the lower level. These facts were later ascertained by Sharman (1942) and Esau (1943), whose observations on the vascular behavior are similar to that of mine, with the exception of the dual nature of the vascular system, which was emphasised by me. In my observations, the trace strands of the first type follow the outward course and join with the inner system, consisting of compound bundles, but never connect with the outer system, which consists of the outermost peripheral bundles. In other words, large leaf trace strands entering the medullary region of the stem and the smaller ones not becoming medullary, both derived from one and the same leaf, constitute two independent vascular systems (the inner and the outer respectively) as diagrammatically shown in Fig. 5. In this respect

the leaf trace behavior in *Zea* is not of the so-called palm type.

Vascular Connection between the Lateral Branch and its Mother Axis

If the dual system of *Zea* is left out of consideration, there is general agreement between *Zea* and most of dicotyledonous plants as to the fundamental way in which the vascular system of a lateral branch connects with the mother axis. As Miller & Wetmore (1946) have pointed out, a prevailing understanding has not been formed for the mode of the vascular connection between the lateral branch and its mother axis. This is true especially in the case of grasses in which the scattered bundles are numerous and the nodal anastomoses are complicated. Thus the previous descriptions given by de Bary (1877) and Bugnon (1920b, 1924), for example, are rather fragmentary. Arber (1930) followed some bundles of an axillary bud toward its mother axis by serial microtome-sections. But even if we can follow downward very exactly a few bundles of a lateral bud, a valid picture of the vascular relationship between the lateral bud and its mother axis cannot be obtained.

In *Zea*, the lateral branch of the simplest vascular construction is represented by the staminate spikelet. The paired spikelets usually have a common short stalk at the base and have been interpreted as a single short shoot subtended by a quite reduced phyllome by Lindstrom (1925), Weatherwax (1927), Cutler & Cutler (1948), Reeves (1950), Nickerson (1954), Galinat (1954) and others. As the vascular connection of the paired spikelets with the mother axis was already described in

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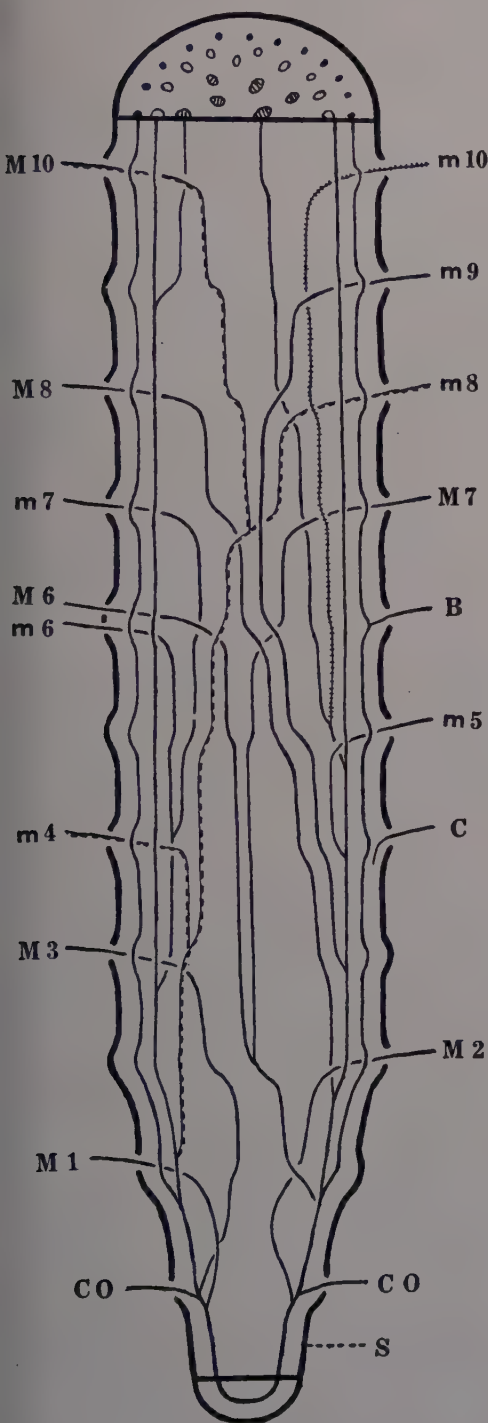
Fig. 5 — Diagram showing the dual feature of the vascular system and the longitudinal course of leaf traces in a vegetative stem with 10 nodes. *M1-M10*, leaf trace strands from the midribs of the first to the tenth leaves respectively; *m4-m10*, those from lateral veins of the fourth to the tenth leaves. (*CO*, trace strand from the coleoptile; *B* and *C*, leaf trace strands of the second and the third types respectively; *S*, mesocotyl.) (Kumazawa, 1940b.)
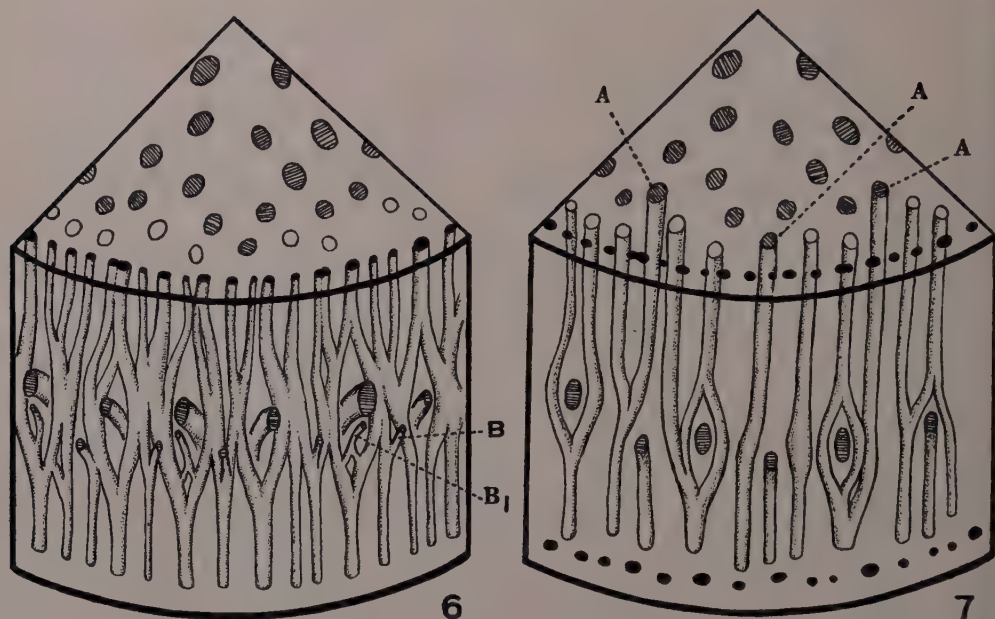
FIGS. 6-7 — Reconstructed model of a part of the vegetative node, showing longitudinal course of the outer vascular system (Fig. 6) and of the inner vascular system (Fig. 7) separately. The nodal anastomosis is not shown. Shaded bundles seen in front are leaf trace strands belonging to the leaf inserted at this node. B and B_1 are small leaf traces which directly connect to the outer system. B_1 indicates the minute trace which is found within the abaxial bundle sheath of the large leaf trace strand figured just above it. The bundles labelled A indicate the leaf traces which have diverged to the location of the inner system at this node. (Kumazawa, 1946).

detail by me (1939, 1940a), it is here diagrammatically shown. The outer and the inner systems are portrayed separately in Figs. 10 and 11 respectively. As seen in Fig. 11, the paired inner branch traces, each of which is derived from the central bundles of each spikelet, connect with a pair of bundles of the inner system of the mother axis at the node or at some distance below it. Some branch traces, derived from the outer system of the fused base of the paired spikelets (Fig. 10), connect with a pair of bundles belonging to the outer system of the mother axis, while the rest of the branch traces descend downward through the mother axis and contribute to its outer system.

It is usual that peripheral bundles of the lateral axis are not found on the adaxial side, and also that those of the mother axis, descending from above and directly facing the lateral branch, are weakly differentiated or die out as indicated by

'x' in Fig. 10 at a level above the node. However, the vascular connection between the lateral branch and its mother axis in Zea is not fundamentally different from that of most dicotyledons with an exception of its dual nature of vascular system. The vascular connection between the secondary staminate rachis and its mother axis is different from that between the paired spikelets and their mother axis in the following respects: (1) there are more numerous bundles both in the secondary rachis and in its mother axis; (2) the inner and the outer systems of both axes are more irregular in arrangement; (3) the outermost peripheral bundles develop not only on the adaxial side of the lateral axis, but also on the side of the mother axis, opposite to the lateral axis; (4) some of the bundles of the inner system enter the mother axis and descend through it without immediately connecting with the inner system of the mother axis at the node.
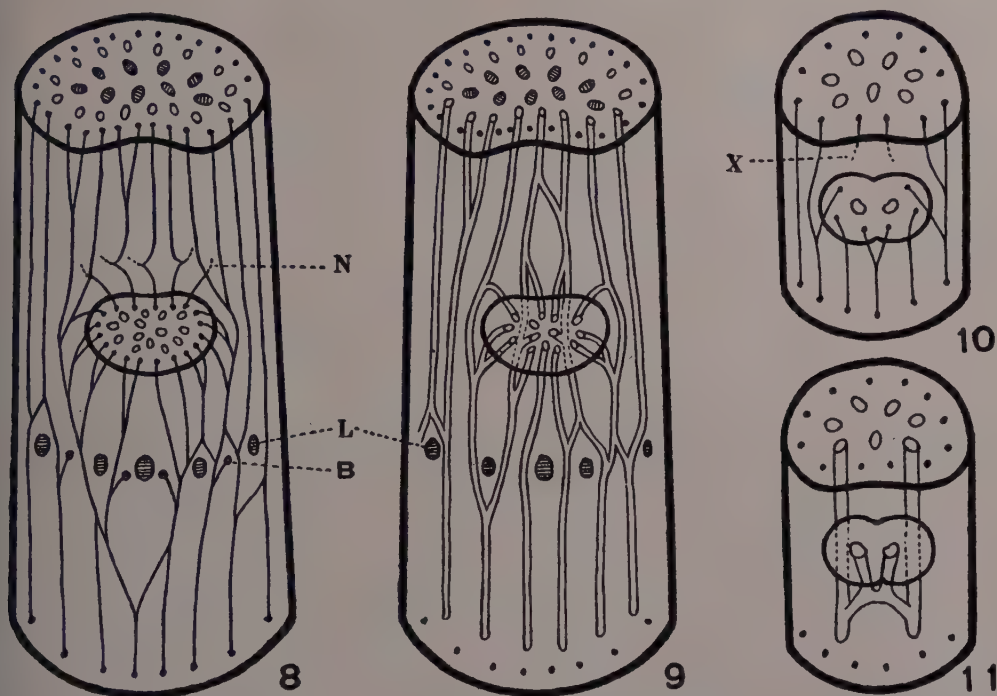
FIGS. 8-11 — Diagrams showing the vascular connection of the axillary shoot — shank — with its mother axis (Figs. 8-9) and that of the fused base of paired staminate spikelets with its mother axis (Figs. 10-11). The outer and the inner systems are shown in separate figures. The base of the lateral organ is shown in front as a cut end. Nodal anastomoses are not indicated except at the parts labelled *N*. *L* and *B* are large and small trace strands from the subtending leaf. As the large leaf trace strands follow an inward course at this node, they are indicated here by the tangential cut end. (Kumazawa, 1958a).

The differences in vascular behavior mentioned above result from the increase of bundles in both axes and are not fundamental. This is also true in the case of the vascular connection between the lateral shoot subtended by a leaf and its mother axis, as described by me in detail in my sixth paper (1958a). In this case (Figs. 8, 9), the behavior of the branch traces is greatly modified and complicated owing to a large number of bundles, the presence of the leaf trace strands, and the development of the nodal plexus. Some branch traces become blended with the nodal plexus or they seem to pass across the centre of the mother axis and join directly with the axial peripheral bundles, which are situated on the side farthest from the lateral shoot. As such branch traces are not found in an early developmental stage of the axillary bud, they are interpreted as secondary modifications caused by the prominent development of a strand of the nodal network which is accidentally in contact with some branch traces.

The lateral branch produces a unilacunar nodal structure similar to that of certain dicotyledons, regardless of its vascular construction, which is complicated in the case of the lateral shoot described above, or simple in the case of the paired spikelets. This is contrary to the leaf which produces a multilacunar node.

Vascular Connection of the Adventitious Root with the Stem

Since the classic works of de Bary (1877) and Mangin (1882), various opinions have been expressed regarding the vascular connection of the adventitious root with
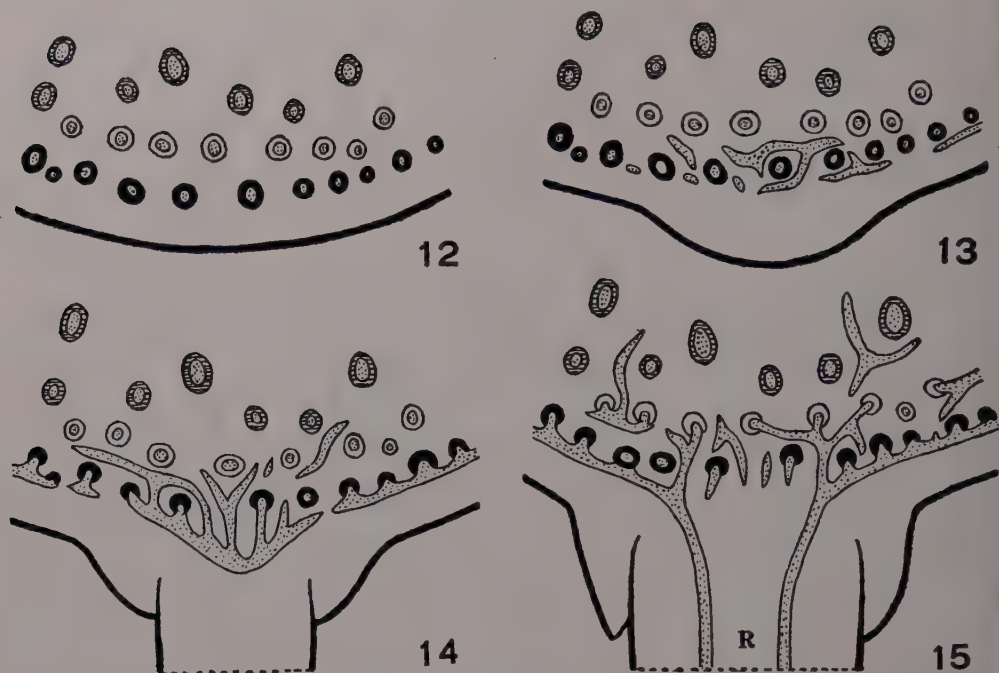
FIGS. 12-15 — T.s. of a part of the stem at various levels from above to below, showing the vascular connection of an adventitious root with the stem. The base of the root (*R*) is longitudinally cut in Fig. 15. Vascular bundles or horizontal procambial strands are stippled. The bundle sheaths of the outer system are indicated in black, those of the inner system in white, and those of the leaf traces are hatched. (Kumazawa, 1958a).

the stem in monocotyledons. On his retted material of *Zea*, Sharman (1942) stated that the root traces join with the nodal anastomosis. Although these observations may be quite valid, the features described are, in my opinion, secondarily modified ones.

As in the case of grasses (Bugnon, 1920, 1924), *Ananas* (Meyer, 1940 ; Krauss, 1948, 1949) and *Allium* (Hoffman, 1933 ; Mann, 1952), my observations (1958a) on *Zea* also show that the adventitious root primordium and its trace strands are initiated at a region near the outer side of the outermost peripheral vascular system.

In the early stages of root development, the root trace strands join with several strands of both the outer and the inner systems as shown in Figs. 12-15. I have not observed a case in which the root trace passes directly across the centre of the stem without connecting with vertical bundles and joins with the peripheral

vertical bundles situated on the opposite side. It was often observed, however, that a few vascular branches, from the root trace strands, elongate horizontally inward, but not so deeply as to reach the centre of the stem.

In short, root trace strands connect with several bundles of both the inner and the outer systems of the stem in the same manner, and the longitudinal vascular courses of both systems are not affected by the insertion of the root trace. Although roots develop in close association with the nodal network, connections between the root traces and the nodal network do not necessarily occur.

Discussion

Since my third paper (1940b) it has been emphasised that two vascular systems, the inner compound bundle system and the outermost peripheral bundle sys-

tem, exist through the cauline axis of *Zea mays* from the base of the stem upward to the tip of the tassel axis. Although the dicyclic feature was discovered in the cob by Reeves (1946, 1950), Laubengayer (1948, 1949) and Cutler & Cutler (1948), Reeves (1953) has not yet pointed out the occurrence of the dual feature of the vascular system in the vegetative shoot. He said that " the occurrence of two systems in the maize cob is a feature by which it differs, apparently or genuinely, from the vegetative stem of maize." In my opinion, the cob differs, very little, from the vegetative shoot in vascular construction. In the cob two kinds of bundles, large and small, are easily distinguished, the former being situated at the central region in an irregular circle, the latter at the periphery; while in the vegetative stem, which has numerous bundles of various sizes, the scattered bundles cannot be divided into groups because of their location, although the smaller bundles are situated at the peripheral region and the larger the bundle, the more centrally located it is. However, it is quite certain that the dual feature occurs also in the vegetative stem. Unless we follow the longitudinal course of each bundle downward through a long distance, we may fail to detect the dual feature of the vascular system in the case of vegetative stem, owing to the fact that numerous leaf trace strands are evenly distributed in the centre of the stem and that the outer and the inner systems are in rather close contact. The cob, therefore, is not essentially different from the vegetative stem in vascular construction.

It is interesting to note that the inner and the outer systems of a lateral axis connect with those of the mother axis, and that the trace strands from a leaf or from an adventitious root connect also with the inner and the outer systems of the mother axis. A similar dicyclic vascular construction in monocotyledons was described by Guillaud (1878) in *Acorus* and by Guillaud (1878) and Gravis (1898) in commelinaceous plants. In the rhizome of *Acorus*, two systems are situated in the vicinity of the peripheral region of the stem. In this case leaf trace strands of small size join directly with the outer system, while those of large size, after entering

the centre of the stem, come back toward the periphery and connect with the inner system as in *Zea*. In the case of *Tradescantia*, the large leaf traces connect with the inner system, which occupies the central region of the stem, but the root trace strands, on the other hand, connect with both systems. According to Guillaud (1878) and Plowman (1906), the vascular bundles of *Scirpus*, *Dulichium* and *Luzula* are dicyclic in arrangement; however, the inner system, constituting the cauline bundles, has no connection with the leaf trace, but joins with the branch traces. If this is true, both systems do not contribute to the vascular supply of the lateral organs, as they do in *Zea*. Further studies, however, are needed in this respect.

I have studied the vascular behavior in some representatives of monocotyledonous families. Although the results of my special observations have not been published, I have found that the scattered vascular systems of some monocotyledonous plants may be interpreted also as having a dual construction, the inner system being situated at the centre of the stem in some species, as in the case of commelinaceous plants, and at the subperipheral region near the location of the outer system in other species, as in the case of *Zea*. The dual vascular construction was ascertained by me in the following plants: *Dendrobium nobile* Lindl., *Epipactis thunbergii* A. Gray (Orchidaceae), *Canna generalis* Bailey (Cannaceae), *Hedychium coronarium* Koen. (Zingiberaceae), *Iris japonica* Thunb. (Iridaceae), *Alstroemeria pulchella* L. (Amaryllidaceae), *Aspidistra elatior* Blume, *Lilium longiflorum* Thunb:, *Polygonatum odoratum* Druce (Liliaceae), etc.

Summary

The series of my papers (1939-1958) regarding the vascular anatomy of *Zea mays* with special reference to vascular behavior has been published in Japanese. In the present paper, therefore, my earlier observations were briefly summarised and some of my findings were emphasized.

Two systems of vascular bundles, one inside the other but independent and

without direct connection with each other, are distinguishable throughout the cauline axis from the base of the stem upward to the tip of the tassel axis. The outer system is represented by the outermost peripheral bundles and the inner system by the compound bundles situated at the sub-peripheral region of the stem.

The branch traces from a lateral axis, such as the paired staminate spikelets, the secondary rachis of tassel and the axillary shoot connect equivalently with both systems of the mother axis. The inner and the outer systems of one lateral axis connect respectively with those of the mother axis. The trace strands from one leaf or from one adventitious root connect also with both

the inner and the outer systems of the mother axis.

The lateral organs of cauline nature produce a unilacunar nodal structure in the mother axis in the same way as seen in the usual dicotyledonous plants.

The nodal anastomoses connect with bundles of the outer and inner systems. They may often connect with the branch traces or with the root traces in the later stage of histogenesis, but the occurrence of vascular connection as such seems to result from the secondary modification.

The scattered vascular bundles found in the stems of many monocotyledonous families may be also interpreted as having the dual pattern and are thus similar to the case of *Zea mays*.

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FERTILIZATION AND EVENTS LEADING UP TO FERTILIZATION, AND THEIR BEARING ON THE ORIGIN OF ANGIOSPERMS

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The main feature distinguishing angiosperms is undoubtedly their mode of reproduction. They owe their success primarily to double fertilization which is the superior form of fertilization since two results of utmost importance are attained through it: (a) the formation of a differentiated embryo, and (b) the production of the secondary endosperm nucleus supplying a highly effective nutrient for the new organism. This peculiar element, the fertilized endosperm, which is formed only in angiosperms, combines polyploidy with heterozygosity, which is possibly the reason why it possesses special nutritive qualities.

It is quite natural, therefore, that speculations on the origin of this dominating plant group were based primarily upon data relating to double fertilization and to the characteristic structural and functional features of the gametophytes and of the sexual elements involved in this process. The whole assemblage of structures

and processes culminating in double fertilization, and especially the organization of the embryo sac, appeared so singular that most writers regarded it as an irrefutable evidence in favour of a strictly monophyletic origin of the angiosperms. According to this view, moreover, the angiosperms are separated from the gymnosperms by an unsurmountable gap as no signs of transition from the latter to the former could possibly be imagined. O. Porsch had made an attempt to find the missing piece of evidence by interpreting the embryo sac as a structure composed of two reduced archegonia; but his theory failed to get recognition, although as will be seen later, there was a positive element in it, namely, the homology between the behaviour of the male fertilizing elements in angiosperms with that in gymnosperms. The most popular theory at present is the so-called Gnetalean theory or the theory of the equipotentiality of the elements of the embryo sac, which was outlined in the early days of plant embryology by Hofmeister and Strasburger. However, this theory, too, is faced with difficulties, as it gives no rational explanation for the structural organization of the embryo sac.

The main trouble seems to me to be that the structures involved in double fertilization are usually regarded as organs or tissues while, because of the extreme reduction which has occurred in the angiosperms, they should be studied primarily on the cellular level; as a matter of fact the constituent elements of the embryo sac or of the pollen grain are merely cells whose behaviour is controlled by the same dynamic principles which underlie cellular behaviour in general. Of course, every particular expression is conditioned by the surroundings operative at the one or the other stage of ontogenesis. The aim of this communication is accordingly an attempt to analyse the phenomena of sexual reproduction in the light of these basic principles of cellular life.

Among the cytological principles some of which were formulated by the pioneers of our science (Hofmeister, Sachs, Hertwig, S. G. Navashin, Strasburger, Haberlandt, Nemec and others) the following are of prime importance.

1. The nucleus (and accordingly the division figure) tends to assume a definite position or " the dynamic centre of the cell " which generally coincides with the centre of the accumulation of the active cytoplasm.

2. The axis of the karyokinetic figure tends to be arranged along the longer axis of the protoplast so that elongated cells generally undergo an equal cross division. If there is no pronounced cellular growth the ensuing division is at right angles to the preceding. This principle is undoubtedly nothing else than an expression of a polarity which is distinctive of any cell.

3. The diverging sister chromatid groups and subsequently formed sister nuclei repel each other. This repulsion ceases with the formation of the partition wall, when principle 1 comes to action, and the nuclei assume the dynamic centres of the sister protoplasts.

4. In bi- or multinucleate cells (coenocytes), nuclear divisions occur synchronously. Mutual repulsion persists between all the nuclei so that they come to lie at the greatest possible distances from each other. In a binucleate coenocyte the nuclei occupy opposite poles; in one which is quadrinucleate there is tetrahedric (or square) arrangement. In a multinucleate coenocyte the nuclei tend to be uniformly distributed so as to form a very regular pattern.

5. The interaction between the nuclei and the surrounding cytoplasm of the coenocyte results in the characteristic radiating organization of the cytoplasm between them (" secondary spindles ") where partition walls may be finally formed according to circumstances.

6. After the mitotic activity in a coenocyte comes to an end the nuclei cease to repel each other and are drifted to a common dynamic centre.

7. By virtue of a mutual nucleo-cytoplasmic interaction the character of the nuclear development depends on the quantity and quality of the surrounding cytoplasm. The growth and the development of the nucleus is not only correlated with the mass of the cytoplasm (" nucleoplasmic ratio ") but its cyclic behaviour also largely depends on definite cytoplasmic properties underlying the synthesis of the

various cellular elements. If the cytoplasm is too scarce and deprived of certain elements, the nucleus enclosed in it never accomplishes its mitotic cycle, and such a cell is incapable of normal self-reproduction.

8. In bi- or multinucleate cells where the nuclei coalesce, nuclear fusion can take place under special physiological conditions whenever the nuclear membranes break down owing to initiation of mitosis, while repulsion between nuclei is absent. This peculiar situation, existing in ageing cells, may be described as a sort of physiological "depression".

An analysis of the organization of the "Normal type" embryo sac shows that it is a result of the operation of the general principles outlined above, rather than a *sui generis* occurrence.

Thus, in the course of the first mitosis in the *macrospore* the sister nuclei diverge to opposite poles of the elongated cell (principles 1, 2 and 3). The second and third divisions in the coenocyte take place synchronously (principle 4) and are oriented at right angles to each other (principle 2); this is the reason why the obligatory quartets of nuclei are formed at the micropylar and chalazal poles of the developing embryo sac. The nuclei composing a tetrahedrical quartet repel each other (principle 3) so that the following final arrangement is inevitably attained in each of them: three nuclei are pushed toward the arch-like apex of the coenocyte while the fourth becomes turned toward the centre which is occupied by a large vacuole. Somewhat later partition walls are formed in the quartet (according to principle 5), i.e. the egg apparatus and the antipodals are formed. Strong vacuolization prevents the formation of a cell wall between the distantly placed fourth nuclei of the quartets (the polar nuclei), resulting in the formation of a large binucleate central cell. With the cessation of nuclear divisions, the polar nuclei soon come to a common dynamic centre (principles 1 and 6) where they may fuse (principle 8) to form the secondary nucleus (Fig. 1).

We thus see that the formation of the embryo sac is an inevitable consequence of the operation of some general cytological principles, which would result in a similar structure in every meristematic cell placed under corresponding conditions. This is clearly evidenced by instances of di- or tetrasporic and especially of aposporic development, as well as by the experimental production of embryo sac like structures. There is no need, therefore, to assume that the "Normal type" embryo sac of angiosperms, despite all its specificity, should have arisen but once in the course of evolution as a unique hereditary structure produced only by the macrospore. The same is true of the special types of embryo sac formation which could have arisen independently and not merely from the Normal type, as is usually assumed. The best case for this point is the occurrence of di- and tetraspory in *Gnetum* (Lotsy, 1899; Fagerlind, 1941). Briefly, it may suffice to state that the type of embryo sac formation would most probably depend upon the specific course of development of the ovule, which modifies the expression of the general principles. As shown previously by the writer (Gerassimova-Navashina, 1954a, b), of prime importance in this connection is the physiological polarity of the ovule, which is correlated with the rate of differentiation and with the food supply. It is most generally manifest in a depression taking place at one of the poles. With the normal type of development the depression first occurs at the micropylar end, which results in the breakdown of the three macrospores. Later on, when the conducting system is developed in the integuments, the relations become reversed, and it is the chalazal end which begins to suffer a depression. Now the type of embryo sac development should depend largely on the relation between the rate of the development of the embryo sac and the differentiation of the food supplying system. There is plenty of evidence supporting this hypothesis and even such specialized types as those occurring in *Oenothera* or *Fritillaria* may be satisfactorily interpreted on this basis (Gerassimova-Navashina, 1954a, b). Of special interest here is the "1+3" arrangement of nuclei in a developing embryo sac of *Fritillaria* etc., which may be most easily understood as the result of an early depression at the chalazal end (reduced activity of the two
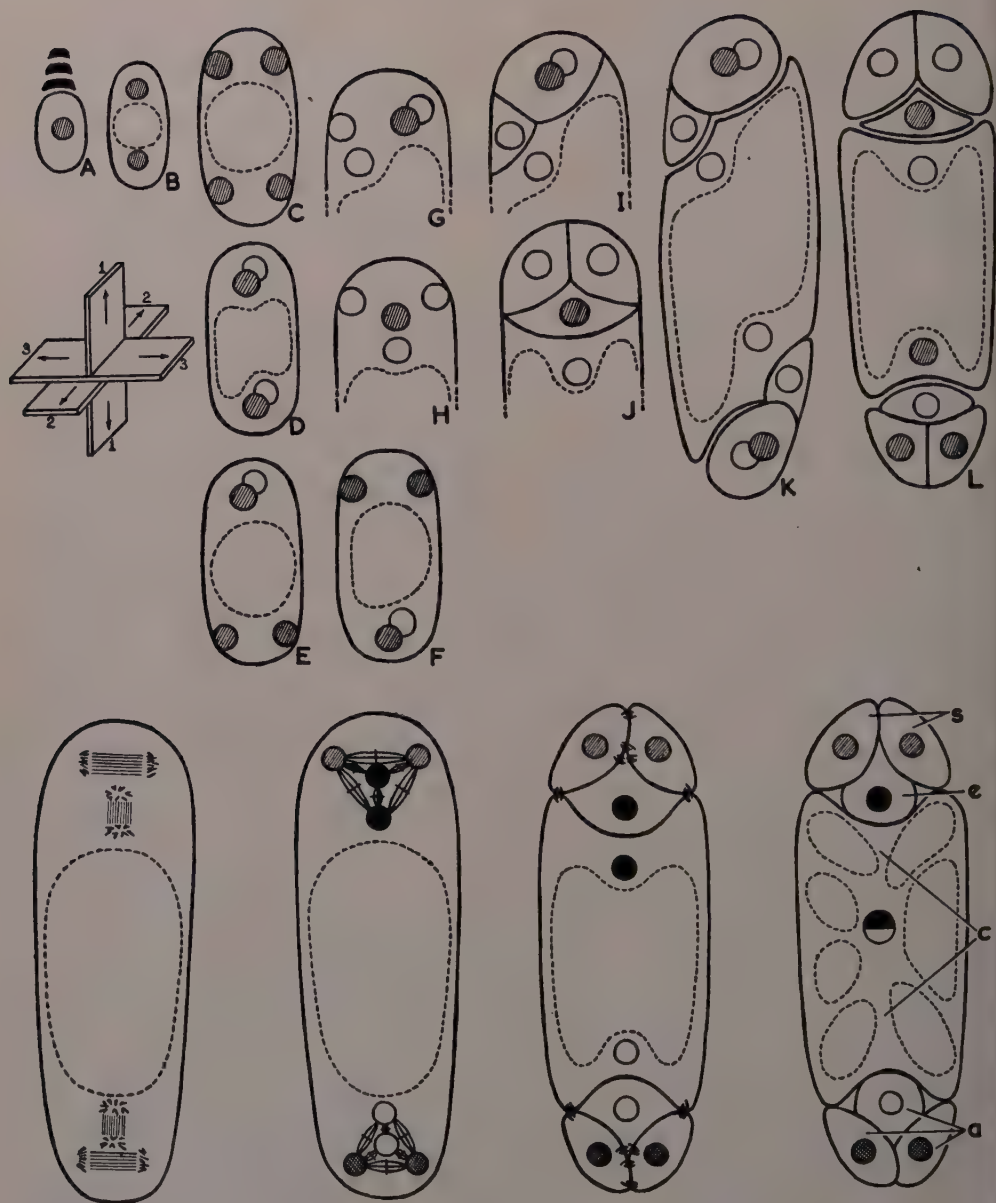
FIG. 1 — (a, antipodals; c, central cell; e, egg cell; s, synergids). Diagram illustrating the development of the normal type of embryo sac. Top: the three nuclear divisions, their orientation and the resulting arrangements of nuclei followed by cell formation. The hatched nuclei are those nearer to the observer. A, the mature macrospore; above the three disintegrated ones. B, the binucleate coenocyte. C-F, the quadrinuceate coenocyte: C-D, square arrangement, face and side view; E-F, cross arrangement, face and side view. G-H, the micropylar tetrahedral quartets of nuclei resulting from the third (last) division, face and side view. I-J, same, after the formation of the partition walls. K-L, embryo sac organization, face and side view. The lower four figures mark the position of the spindles at the third division and the organization of the embryo sac; the pairs of sister nuclei are shaded identically (face view).

lower nuclei as a disturbance of principle 4 occurring in four-nucleate coenocytes) which leads later to the union of the three dividing chalazal nuclei, the " Bambacioni effect " (our principle 8) resulting in the formation of a " secondary four-nucleate " coenocyte.

The male gametophyte of angiosperms — the pollen grain — is distinguished by its morphological reduction which has here gone to the highest possible extent. In all the representatives of the order it consists of two strikingly different cells, the siphonogenic (vegetative) and spermiogenic (generative). The spermiogenic cell receives a small amount of cytoplasm which is also almost deprived of active elements such as mitochondria. The siphonogenic cell, on the other hand, receives nearly all the active cytoplasm of the microspore. This difference, which is the result of an asymmetrical division in the microspore, undoubtedly decides upon the activities of the two cells: while the spermiogenic cell is incapable of any growth, the siphonogenic cell is distinguished by its extremely strong physiological activity, resulting at first in marked growth of its metabolic nucleus and nucleolus (the latter is often larger than the entire spermiogenic nucleus) and in the accumulation of storage material; later on it displays an amazing energy of growth, while developing the pollen tube. The vigorous growth of the pollen tube is an outstanding feature of the angiosperms which no doubt played an important part in their success. The two cells which thus possess, so to say, contradictory properties form a peculiar unified system called the " fertilizing system " (Gerassimova-Navashina, 1951) which is indispensable for double fertilization.

Of much debate was the question as to the morphological nature of the two components of the male gametophyte of angiosperms. In my opinion there is no reason to assume that the pollen grain is, or contains a primordial antheridium (Meyer, 1953; Modilewsky, 1953; Battaglia, 1951). Comparative studies reveal, on the contrary, that the very first gametophytic cell which should be regarded as a homologue of a prothallial cell, directly becomes the generative element in the angiosperms

while its robust sib is nothing other than an apical cell (Wettstein, 1911; Ferguson, 1904). As this assumes in angiosperms an entirely new function connected with the development of a highly efficient structure, the pollen tube, I prefer the term " siphonogenic " to qualify it (Gerassimova-Navashina, 1951).

The different course of development of the two components of the pollen grain becomes the basis for their fundamentally different functions. The spermiogenic cell which mainly contains the nuclear material undergoes a curtailed division giving rise to two sperms which are unable to complete their mitotic cycle and remain in a state comparable to a continued telophase. If they do not remain linked together to form a twin structure, they vigorously repel each other, like sister chromatid groups or sister nuclei (principle 3). In those species whose sperms are rich in cytoplasm (e.g. *Butomus*) the nuclei which are at first close to the poles migrate to the centers of their respective cells during the maturation of the sperms. We thus see that the behaviour of the sperms conforms to the general principles of mitotic behaviour, and they cannot possibly behave otherwise because of their intracellular mode of life, which is another striking characteristic of angiosperms. The pollen tube developing from the siphonogenic cell has to do a tremendous work, i.e. delivering the pair of sperms to the embryo sac. To accomplish this it must cover a path which is sometimes several thousand times the diameter of the pollen grain. The spermiogenic cell or the sperms (if they are formed prior to the germination of the pollen grain) have to travel all along the growing pollen tube till a certain point near the apex is attained. The nature of this movement has also been the subject of much discussion.

Detailed microkinematographic studies on several suitable species (Navashin *et al.*, 1959) make one think that the elements in question are transported by the streaming cytoplasm rather than by their autonomous motility. The reason for this conclusion lies in the fact that the acropetal flow of cytoplasm obviously differs in its physico-chemical properties from the basipetal one, the generative elements

showing a stronger attraction to the former than to the latter. Thus it may be assumed that the behaviour of the spermiogenic cell or the sperms tending to assume a definite position within the protoplast does not differ from that of any nucleus (principle 1); the main difference consists in the fact that, in the pollen tube one can more easily see the source of motion — the streaming of the cytoplasm which is not always so when ordinary tissue cells are observed. However, if one thinks of the cells of the staminal hairs of *Tradescantia* or the rotation of nuclei in meristematic cells, this suggestion will appear at least probable. The main peculiarity of the pollen tube consists in the longitudinal orientation of cytoplasmic currents, while in the majority of cells with centrally located nuclei there are radial cytoplasmic streamings (i.e. from the nucleus to the periphery and in the opposite direction).

We have thus seen that in the course of evolution the male and female gametophytes of angiosperms had suffered extreme morphological reduction and ultimately attained the cellular level of organization. This process which is characteristic of plant evolution in general is based upon an acceleration of development owing to which the sexual functions are realized at a progressively earlier ontogenetic moment. Thus, if in the gymnosperms more than 10 cell generations are necessary to initiate the formation of archegonia in the angiosperms, the organization of the embryo sac is achieved within 3 cell generations and one of the cells of the primordial prothallus directly becomes an egg. Thus the process never goes as far as archegonium formation. Acceleration is still stronger in the male gametophyte where, as we have already seen, the very first cell of the prothallus proves to be the spermiogenic one. This general trend of evolution which is characterized by an enhancement of development, leading to a progressively earlier functional maturity, was pointed out as early as 1909 (Coulter, 1909). Recently special attention has been paid to this question (Romanov, 1944; Fagerlind, 1944; Takhtajan, 1948, 1950; Battaglia, 1951; Gerassimova-Navashina, 1954a,b, 1958). We find it most fitting to refer

this phenomenon to acceleration in the sense given to this term by Sewertzoff (1939); it is less suitable to classify it as neoteny (Romanov, 1944; Takhtajan, 1948) which is a different phenomenon (signifying the occurrence of adult characteristics in larvae).

We shall now attempt to discuss the central problem of double fertilization. As already pointed out, this problem is intimately linked up with that of the nature and origin of the embryo sac and pollen grain. Although the archegonium theory which aimed to tie up the embryo sac of angiosperms with the female gametophyte of gymnosperms proved a failure on the whole, it might be of service if that part of it which deals with double fertilization is carefully taken into account. Numerous known instances of the fertilization of the ventral canal nucleus in gymnosperms should undoubtedly be taken as a partial manifestation in lower plant groups of the same principles which attained their full expression in angiosperms. In other words, the conditions, whose full development results in double fertilization under the circumstances existing in the angiosperms, can also be discovered in the gymnosperms. These may be summarized as follows (Gerassimova-Navashina, 1954a, b): (i) a paired condition and functional equivalence of sperms; (ii) the incomplete mitotic cycle peculiar to sperms; (iii) a mutual repulsion of sperms by virtue of the mitotic activity still retained by them; and (iv) a definite cytoplasmic organization existing between the neighbouring female nuclei.

As may be seen from the foregoing data no essentially new elements have appeared in the angiosperms. However, such conditions were developed in this group, which allowed the elements already present to function to a full extent. These conditions consisted in the first place in the reduction of the embryo sac which attained the cellular level of organization, and in a full functional development of the pollen tube.

If we had more space, we could analyze the historical transformation of these elements in the lower groups on the basis of the available information (Ferguson, 1904, on *Pinus*; Land, 1902, 1907, on *Thuja* and *Ephedra*; Herzfeld, 1922, on *Ephedra*;

Hutchinson, 1915, on *Abies*; and others); however, we should restrict ourselves to the following brief annotations. The paired condition of sperms is already well pronounced in gymnosperms; this could not possibly disappear, since the sperms are always products of recent division, even in gymnosperms; the reason lies probably in their reduced vitality the cause of which has been explained above. The above mentioned strong development of the pollen tube undoubtedly proved decisive for the simultaneous delivery of the two sperms to the female sphere.   Finally, the mutual repulsion of the sperms composing a pair is quite pronounced in gymnosperms.

According to the cytological hypothesis of double fertilization as developed by the writer (Gerassimova-Navashina, 1947, 1954b, 1957) the fundamental element of double fertilization consists in the directed divergence of the components of a pair of sperms introduced by the pollen tube into the specifically organized cytoplasm of the two neighbouring female cells.   In so far as all the elements of this behaviour, which at first glance seem to be entirely unprecedented, are already present in lower groups, for the realization of double fertilization, a reduction of the structure of the embryo sac leading to a cellular level of organization was sufficient.   In other words, the origin of the embryo sac corresponded with the origin of double fertilization.   With a gymnospermous level of organization of the gametophyte the process of fertilization could not yet assume the form typical of angiosperms.   Further reduction of the organization of the gametophyte, which was connected with its still earlier maturation, finally made possible a regular functioning of the two sperms.   This important acquisition was the result of a progressive development of those elements which were present in lower groups where they could, however, come into action only in exceptional cases. When a certain level of organization was attained, they acquired full importance. This brought into play a whole assemblage of events, which ultimately resulted in a superior form of fertilization.   In so far as there are no grounds to regard the embryo sac to be a *sui generis* structure which could arise only once and at a single locality, it may be suggested that the premises of double fertilization arose repeatedly every time when circumstances made possible the manifestation of those general cytological principles which have been discussed above.

We thus come to a conclusion that there is nò reason to believe that embryological evidence creates a wall or a hiatus between the gymnosperms and angiosperms or may be consistently regarded as a testimony to a strictly monophyletic origin of angiosperms.

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SEASONAL VARIATIONS IN THE SHOOT APEX OF *CEPHALOTAXUS DRUPACEA* SIEB. ET ZUCC.

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According to Koch's (1891) account of the histological organization of the shoot apex of *Cephalotaxus pedunculata*, there is a small group of apical initials which undergo periclinal and anticlinal divisions that contribute to the subapical initials and to the usual flanking zone. Beneath the subapical initials there is a large zone of irregularly arranged cells which form the pith and possibly the inner portion of the flanking tissue. Korody (1937), Kemp (1943), Sacher (1954) and Parke (1959) have, however, shown that in the same coniferous species the shoot apex may vary markedly in size and structure during different seasons of growth. Since Koch never mentioned the date of collection of his material, his observations do not enable any comparisons.

An attempt is made here to describe the various changes during the different months of a year, in the shoot apex of female trees of *Cephalotaxus drupacea*, the material of which was collected from the Forest Research Institute, Dehra Dun.

The data presented concern lateral apices which do not, however, differ in any important way from the terminal apices except that they are slightly smaller. In the present description the term shoot apex refers to the meristem above the last lateral appendages only (cf. Parke, 1959).

The material was fixed in formalin-acetic-alcohol. Opposite sides of the buds covered with scales were trimmed before fixation. The customary methods of dehydration and imbedding were followed. Sections were cut 8-12 microns thick and stained with safranin and fast green.

Observations

EXTERNAL MORPHOLOGY — *C. drupacea* is strictly a dioecious, evergreen small tree. The leaves are small, strap-shaped, and

acute, and arranged in a decussate and two-ranked fashion. The new shoots emerge from the resting buds in the month of March and elongate fully in about a month. Their apices produce numerous small bud scales which persist even after the unfolding of the bud. Lateral growing points covered by scales arise in the axils of two or three of the uppermost leaves. In October the young shoots show an apical and two or three lateral buds, all covered by scales. These are the winter or the resting buds. Shoots emerge from these buds in the spring. The apical bud continues the growth of the main axis while the lateral buds produce lateral branches. Thus, the buds on a shoot do not function in the same year in which the shoot has emerged. The branches arise in an opposite or alternate fashion depending on whether both or only one of the leaves of a node bears the bud. Since the branches are very close to the apex and to each other, they present a whorled arrangement (Dallimore & Jackson, 1954).

HISTOLOGICAL ORGANIZATION — Four cytohistological zones can be differentiated in the shoot apex: (i) the apical initials, (ii) the subapical initials, (iii) the flank meristem and (iv) the rib meristem (Fig. 1). The apical initials occupy the tip of the apex and in a longitudinal section consist of three to five lightly-staining large cells with non-vacuolate cytoplasm and large nuclei. These initials divide both periclinally and anticlinally so that a typical tunica layer is absent. The anticlinal derivatives form the epidermis and the periclinal derivatives contribute to the subapical initials which form a group of cells having large size, more conspicuous nuclei and vacuolate cytoplasm taking a light stain. The subapical initials are characterised by irregular divisions and angular thickenings; occasionally one of these cells becomes unusually large. The derivatives of the subapical initials on the flank and at the base produce the flank and the rib meristems respectively. The flank meristem comprises a few layers of densely-staining small cells. Owing to irregular divisions the layers are not stratified. The cortex and the vascular tissue of the mature stem is derived from

this zone. The rib meristem has elongated, highly vacuolated and lightly-staining cells. These are arranged in longitudinal files and are characterised by predominantly transverse divisions. The derivative cells form the pith.

SEASONAL VARIATIONS — The shoot apex shows marked variations in activity, size, topography and histological organization. There are four periods:

(i) Resting period, lasting from the second week of December to about the fourth week of February,

(ii) Period of bud elongation, lasting from the end of February to the beginning of April,

(iii) Period of bud scale formation, lasting from the second week of April to mid-July,

(iv) Period of foliage leaf formation, lasting from middle of July to the second week of December.

Variation in Activity — The resting bud consists of a young telescoped shoot on which all the leaf primordia have differentiated and is surrounded by numerous scales (Fig. 2). A plate of thick-walled cells, termed as ' crown ' by Lewis & Dowding (1924), extends across the base of the bud except in the area occupied by the vascular tissue. The apical dome is relatively high and broad.

During the period of bud elongation, the bud scales open, the enclosed young shoot elongates and the already existent leaf primordia expand into mature leaves. In a longitudinal section through a very long shoot, many ' crowns ' can be seen at various levels. The distance between the successive crowns represents one year's growth of the axis. With the onset of elongation the diameter of the apical dome decreases markedly while its height remains almost the same.

All the bud scales (26-33) arise in acropetal succession and complete their development during the period of bud scale formation, i.e. mid-April to mid-July (Figs. 3-5). Externally no change is visible in the bud after this period. Although initially the apex increases in diameter, by the middle of June it has decreased both in diameter and height and by the middle of July the apex becomes very narrow and low (Fig. 5).
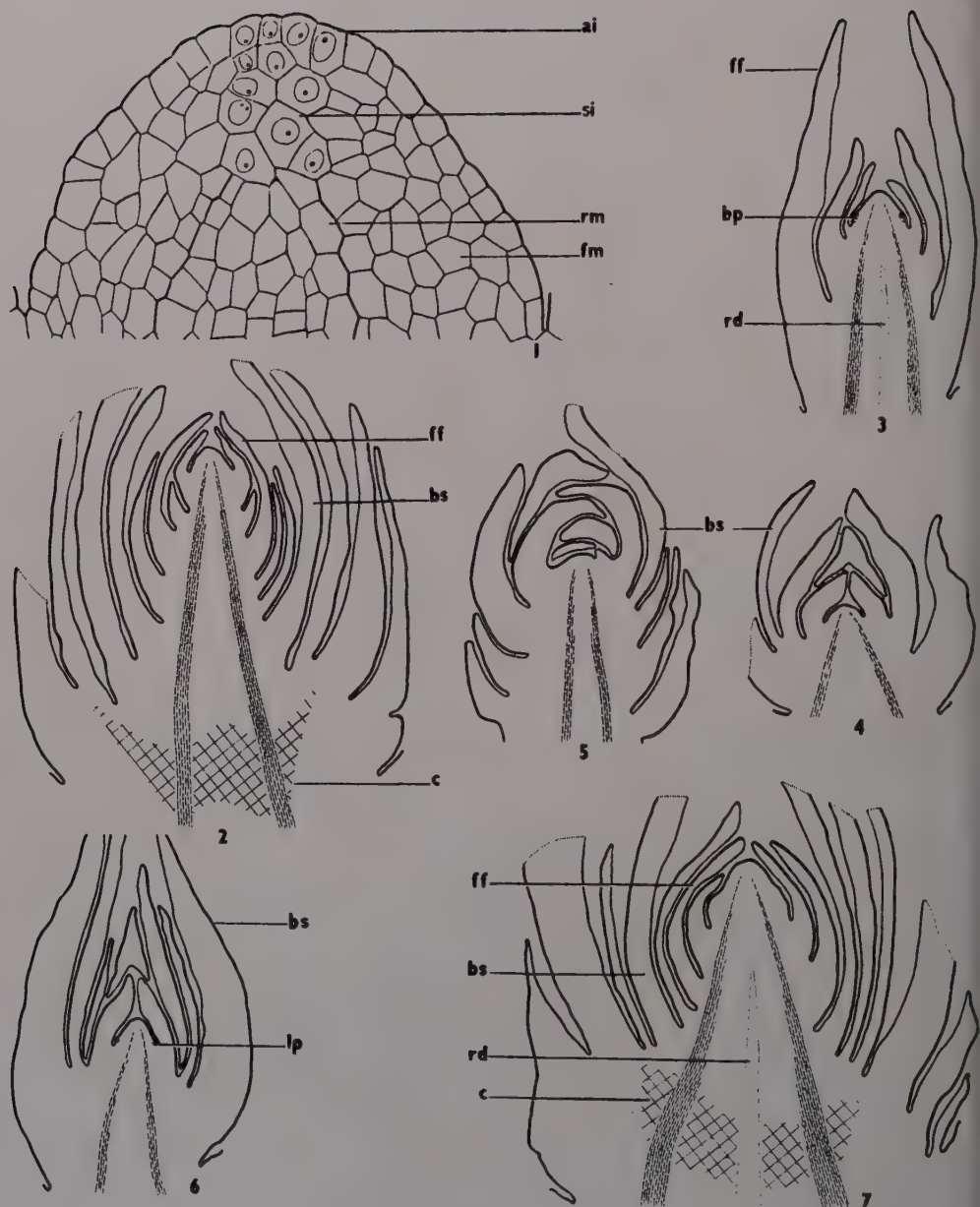
FIGS. 1-7 — (ai, apical initials; bp, bud scale primordium; bs, bud scale; c, crown; ff, foliage leaf; fm, flank meristem; lp, leaf primordium; rd, resin duct; rm, pith rib meristem; si, subapical initials).   Fig. 1. L.s. shoot apex showing apical initials (ai), subapical initials (si), flank meristem (fm) and rib meristem (rm).   × 412.   Figs. 2-7.   Longisections apical buds in different growth periods.   The bud represented in Fig. 2 is in the resting period.   Figs. 3-5. Buds at the beginning, middle and the end of the period of bud scale formation.   Figs. 6, 7. Beginning and middle of period of foliage leaf formation.   × 40.
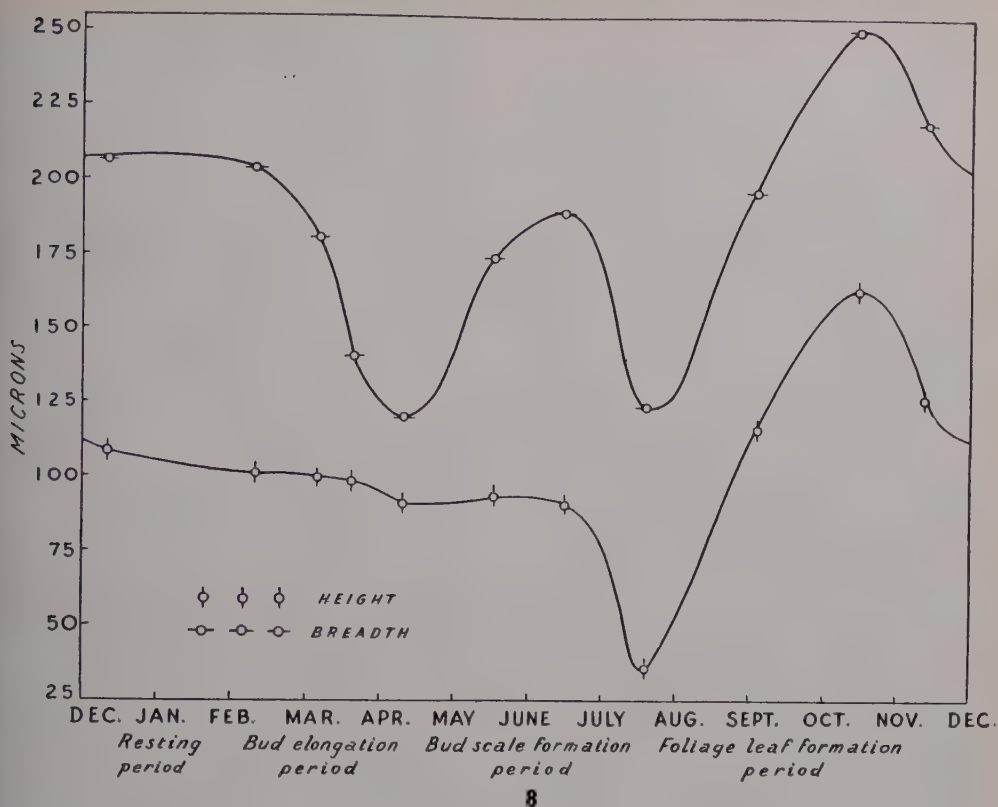
FIG. 8 — Graph showing variations in the dimensions of the shoot apex during a year. Each point on the graph represents the average measurements of 10-12 apices.

The leaf primordia (20-24 pairs) are laid down in the period of foliage leaf formation (Figs. 6, 7). The scale-like leaves in whose axil the female cones arise are the first to differentiate. Leaf primordia are laid down at a rapid rate from the middle of September to about the third week of November. Later the activity slows down considerably. In the middle of the period the apex attains its maximum dimensions. Usually the buds containing the female cones have a smaller vegetative apex. Figure 8 shows the variations in the dimensions of the shoot apex during a year.

Variations in Histological Organization — During the resting period the cells of the apex are inactive and the various zones are not clearly demarcated (Fig. 9). However, the surface layer is very distinct since periclinal divisions are almost absent

from it (Fig. 9). The cytoplasm in the various zones is dense, cells are small and the cell walls are thick. There are three to five subapical initials in longisection, about four flank meristem layers and five to seven layers of rib meristem (Fig. 9).

In the period of bud elongation also the cells of the apical meristem remain inactive and the surface layer continues to be distinct (Fig. 10). The cytoplasm in the cells of the apical meristem takes a light stain and the cell walls are thin. The cell size increases somewhat. The number of subapical initials decreases and the extent of the rib meristem remains the same as for the last period but the unstratified flank meristem is reduced to just two or three layers (Fig. 10).

With the advent of the period of bud scale formation the apex becomes active. The surface layer is no longer quite distinct
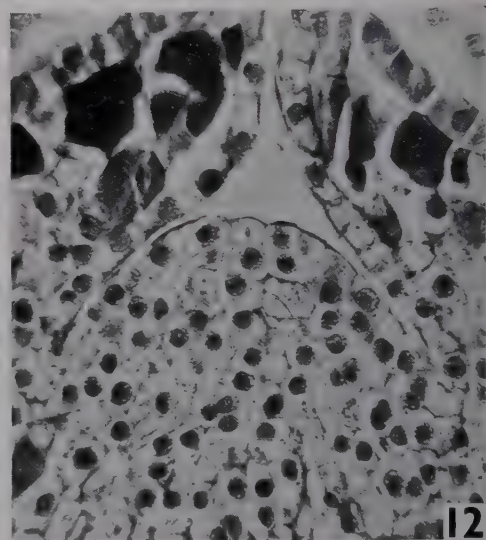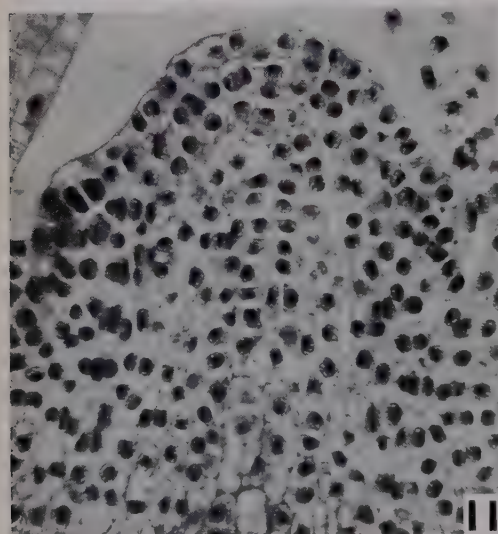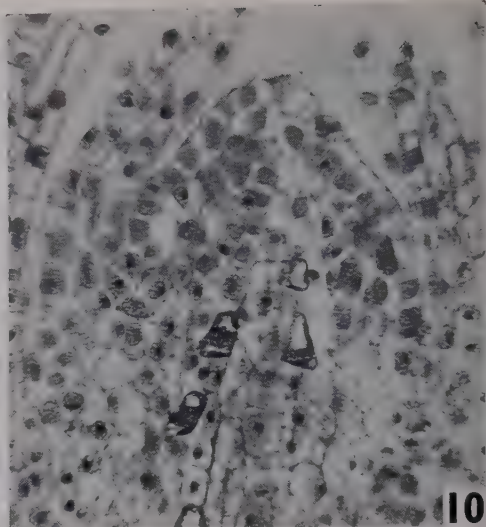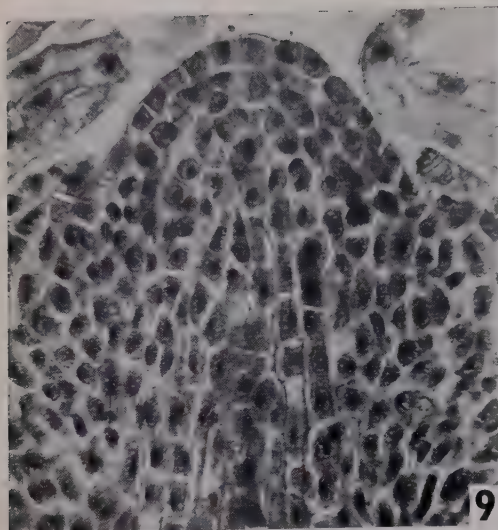
FIGS. 9-12 — Longisections of shoot apices in the resting period (Fig. 9), bud elongation period (Fig. 10), and beginning and middle of the period of bud scale formation (Figs. 11, 12). × 238.

since many periclinal divisions occur in it (Fig. 11). The cytoplasm becomes dense and cell size decreases markedly but no change was observed in the thickness of the cell walls. There is a slight increase in the number of subapical initials and the width of the flank meristem increases to half a dozen layers but the extent of the rib meristem remains more or less cons-

tant. As bud scale formation advances the cell size increases considerably but the extent of the various zones decreases (Fig. 12) until finally the subapical initials (in longisection) consist of only two or three cells and the flank meristem is three or four layers thick (Fig. 13). The cytoplasm becomes thin in middle of the period but again appears thick
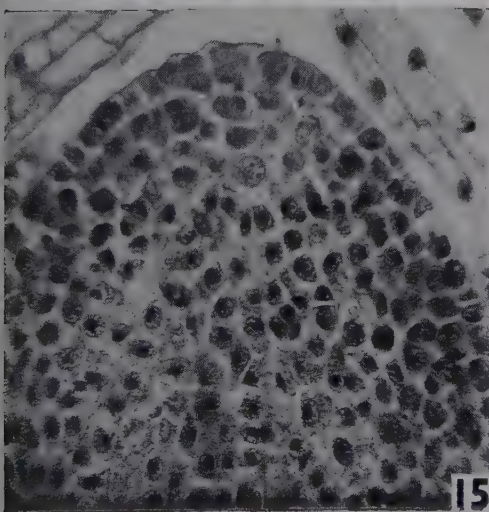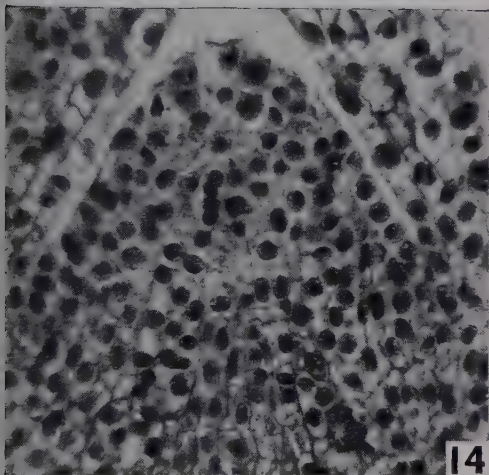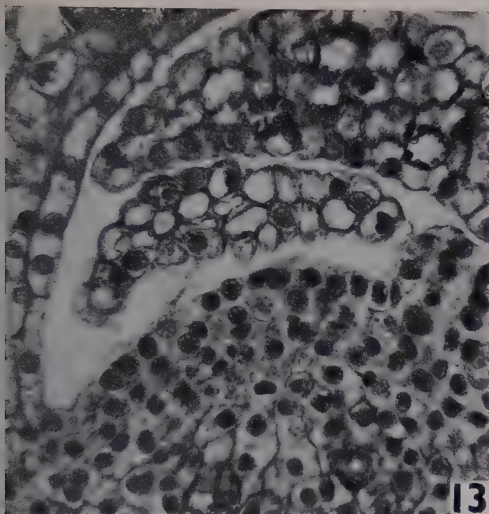
towards the end of the period. Initially cell divisions are frequent but become rare toward the close of this period. The zonation remains clear throughout.

The small ' residual ' apex seen at the close of the last period shows a marked increase in activity and develops into an elevated cone. The leaf primordia originate at its base (Fig. 14). The extent of the various zones and the cell size in general increase and the cytoplasm becomes less dense. The subapical initials increase both in number (7-9, as seen in a longitudinal section) and size, their cytoplasm is more vacuolated, and the walls are thinner. With the advance of this period the cell size decreases (Fig. 15), the cytoplasm becomes dense and the cell walls become thick by the time the resting period is reached. Cell divisions are common and the zonation distinct throughout the period.

At the beginning of September some cells at the base of the new shoot undergo a few transverse divisions and soon become thick-walled to form the conspicuous plate or crown (Fig. 7).

Discussion

The shoot apex completes a cycle of increase and decrease in diameter during the period of bud scale formation. A similar cycle, with reference to the dimensions of the shoot apex, does not occur during any other period. The height of the apical dome shows only a single decrease and increase per year. However, the diameter shows two minima and maxima in the same period. The available data are insufficient to explain the significance of these facts.

Although there is a considerable variation in the details of cellular organization of the apex during different periods, the basic pattern remains the same. During the

Figs. 13-15 — Longisections of shoot apices at the end of the period of bud scale formation (Fig. 13), beginning and middle of the period of foliage leaf formation (Figs. 14, 15). × 238.

months of April-July (period of bud scale formation) the cells are small and thick-walled, with thin cytoplasm. But during July-December (period of foliage leaf formation) the cells are larger and thin-walled, and have dense cytoplasm. The constancy of these details and the type of lateral appendages produced in a season suggest a correlation between the season, the cytohistological organization of the apex and the nature of the append-ages produced. Conclusive proof for such a hypothesis can come only from other fields like an *in vitro* study and a cytochemical analysis of the apical bud.

For the shoot apex of *Cephalotaxus* Johnson (1951) remarked: " Two points should be clarified: first, would the modern histological technique show this central zone to be equivalent to the central mother cell zone of cycads and *Ginkgo*, and second, is there a pith rib meristem in strong leading shoots ? " Present obser-vations show that the pith rib meristem (central zone of Koch, 1891) is not equi-valent to the central mother cell zone of cycads and *Ginkgo*. In the latter the central mother cell zone produces the pith rib meristem whose derivatives form the pith, while in *Cephalotaxus* this is pro-duced directly from the central zone (= rib meristem). A rib meristem is present in all the apical buds. Trans-verse divisions are predominant in the pith rib meristem so that its cells are arranged in files. Koch's (1891) obser-vations, describing this as a zone of irregularly arranged cells, appear to be incorrect.

According to Johnson (1951) the removal of *Cephalotaxus* from the Taxa-ceae is supported by the structure of the shoot apex which is similar in *Taxus* and *Torreya* but quite distinct from that of *Cephalotaxus*. However, a comparison of the histological organization of the shoot apex of *Cephalotaxus* (present work) and *Torreya* (Kemp, 1943) re-veals that the two are similar in the following respects: (i) both show the same four cytohistological zones namely the apical initials, the subapical initials, the flank meristem and the pith rib meristem, (ii) the derivation of the three lower zones

from the apical initials is similar, and (iii) the characteristics of the various zones are also identical. In fact, the structure of the two apices does not differ in any important respect and if this alone were considered, there would be no justification for removing *Cephalotaxus* from the Taxa-ceae. Johnson's (1951) remark only indicates that he was misled by the pre-vious incorrect knowledge of the shoot apex of *Cephalotaxus*.

Kemp (1943) recognized three growth periods in the shoot apex of *Torreya* as against four in *Cephalotaxus* (present work). The apex of both behaves very similarly during different months of the year, but in *Torreya* the period of bud scale formation is continuous with that of foliage leaf formation and, therefore, the two processes have been described under ' new bud formation period '. In *Abies* (Korody, 1937; Parke, 1959) and *Pinus* (Sacher, 1954) also there are three growth periods but in these the bud elongation is continuous with bud scale formation. In *Cephalotaxus* the four periods — rest, bud elongation, bud scale formation and foliage leaf formation — are distinct from each other.

As in *Torreya* (Kemp, 1943) and *Abies* (Korody, 1937; Parke, 1959) the pattern of zonation in *Cephalotaxus* is not as well marked during the resting period as during the phase of active growth.

In general the shoot apex of *Cephalo-taxus* resembles that of other conifers (except the araucarians which seem to show a discrete tunica layer; *see* Johnson, 1951; Griffith, 1952) in having the usual four cytohistological zones. The tunica-corpus concept is not applicable in *Cepha-lotaxus* but the apex can be differentiated into the various zones which are ultima-tely derived from the apical initials.

Summary

Cephalotaxus drupacea is strictly dioe-cious and grows as a small cultivated tree in Dehra Dun. The leaves are linear and acute and are arranged in an opposite, decussate and two-ranked fashion. Soon after the elongation of the shoot, its apex becomes covered by scales and forms a resting bud. The branches arise in an

opposite or alternate manner near the apex of the previous year's shoot.

Lateral shoot apices from female trees were studied. Four cytohistological zones could be demarcated: (i) the apical initials, (ii) the subapical initials, (iii) the flank meristem and (iv) the rib meristem. Every year the shoot apex passes through the following four periods: (i) the resting period, from second week of December to the fourth week of February, (ii) the period of bud elongation, from end of February to the beginning of April, (iii) the period of bud scale formation, from mid-April to mid-July, and (iv) the period of foliage leaf formation, from mid-July to mid-December. The activity, size, topography and details of the histological organization of the shoot apex vary markedly during the different periods.

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THE LIFE HISTORY AND SYSTEMATIC POSITION OF *CEPHALOTAXUS DRUPACEA* SIEB. ET ZUCC.

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Introduction and Previous Work

The genus *Cephalotaxus* includes six species (Pilger & Melchior, 1954) growing from Eastern Himalayas to Japan in sub-tropical forests. The plant is a strictly dioecious, and small evergreen tree. Eichler (1889) placed the genus in the Taxaceae while Neger (1907) erected a new monogeneric family Cephalotaxaceae for it. Pilger (1916, 1926) assigned another genus, *Amentotaxus*, to the family. Pulle (1937) included both Taxaceae and Cephalotaxaceae under the order Taxales. Florin (1931, 1938-45, 1948, 1954) has pleaded not only for the elimination of *Amentotaxus* from the Cephalotaxaceae but considers this family as having no

alliance with the Taxaceae which should form a separate class (Taxales or Taxineae) of gymnosperms. According to Takhtajan (1953), on the other hand, the Taxaceae and Cephalotaxaceae are closely related to each other.

Worsdell (1900, 1901) worked on the vasculature and differentiation of the cones of *Cephalotaxus fortunei* and gave interpretations on their morphological nature. A brief investigation of the vasculature of the female cone was also done by Sinnott (1913). Hirmer (1936) and Dluhosch (1937) gave a more detailed account of the development and structure of the male and female cones. More recently Nozeran (1949, 1955a, b, c) and André (1956) have studied the organization and morphology of the cones.

Strasburger (1879) investigated the development of the embryo after the cellular stage in *C. fortunei*. He described the behaviour of the primary suspensor and showed that the secondary suspensors originate from the embryonal mass. After working out the structure of the genus, Van Tieghem (1891) remarked that *Cephalotaxus* is closely allied to *Ginkgo*. This mistake was, however, soon corrected by the discovery of swimming spermatozoids in *Ginkgo* (Hirasé, 1895). Sokolowa (1890) published a voluminous paper on the endosperm formation in several gymnosperms including *Cephalotaxus*. He figured a thin membrane around the female gametophyte of *C. fortunei*, but Thompson (1905) denied its presence in *C. mannii*. Proembryo development was studied for the first time by Arnoldi (1900). He recorded that in *C. fortunei* the pollen tube contains a large body cell, a stalk nucleus and a tube nucleus; the body cell divides to form two sperm cells; the ovule is pollinated during the first year of its growth and the female gametophyte develops in the second year; the egg nucleus is not separated from the ventral canal nucleus by a cell plate; the intranuclear spindle of the first division of the zygote nucleus may be oriented in different ways; the four nuclei of the proembryo are variously distributed in the cytoplasm; and a ' cap cell ' is present in the proembryo. Coker (1907) examined the various stages of ovule development in *C. fortunei*

starting from the resting stage to the formation of a proembryo. According to him prothallial cells are absent in the pollen grains which are two-celled at the time of shedding, the body cell divides to form two unequal sperm cells, following pollination the ovule rests at the megaspore stage during the course of the winter, the female gametophyte proliferates above the neck of the archegonium, the ventral canal nucleus is ephemeral but sometimes persistent, wall formation in the proembryo takes place at the 16-nucleate stage, and rarely two cap cells are present in the proembryo.

An account of the gametophytes, fertilization and embryo of *C. drupacea* was given by Lawson (1907). He noted that the shedding of pollen grains begins in March and extends for three weeks, the body cell divides to form two equal male nuclei, there are no nutritive cells around the female gametophyte, the archegonium is surrounded by a single layer of jacket cells, and rarely the second male nucleus is present in the upper part of the proembryo.

The seedling structure of *C. pedunculata* was investigated by Hill & de Fraine (1908).

Buchholz (1925) worked out the later stages of embryogeny of *C. fortunei*. Sugihara (1947) gave a short account of the male gametes of *C. drupacea*. He differed from Lawson (1907) in describing the presence of two unequal male cells. Favre-Duchartre (1956, 1957) has contributed to the study of reproduction in *C. drupacea*. He observed that the body cell divides into two equal sperm cells, both of which penetrate the archegonium, rarely a two-nucleate egg is present, the supernumerary male nucleus in the upper region of the proembryo divides mitotically as well as amitotically and the binucleate egg is fertilizable. By using the Feulgen nuclear reaction he could distinguish the degenerating ventral canal nucleus at the micropylar end of some two-nucleate egg cells, thereby showing that the two large nuclei in the egg were the product of division of the egg nucleus alone. Khoshoo (1957a, b) made thorough studies on meiosis in *C. drupacea* var. *pedunculata* and Kaur (1958) has published a brief note on its embryology.

The insufficient work and the many controversial aspects of its systematic position aroused my interest in *Cephalotaxus*, one species of which is easily available in the Forest Research Institute, Dehra Dun.

Material and Methods

Male and female cones of different ages were collected from the trees cultivated in the arboretum of the Forest Research Institute, Dehra Dun, and fixed in formalin-acetic-alcohol (formalin, 5 cc; acetic acid, 5 cc; 50 per cent ethyl alcohol, 90 cc) after proper trimming. Nawaschin's fluid proved superior for fixing female gametophytes at the archegonial stage. The female gametophytes (which were to be later stained with Feulgen) were fixed in chrome-acetic solution (10 per cent aqueous chromic acid, 7 cc; 10 per cent aqueous acetic acid, 10 cc; distilled water, 100 cc) while the microsporangia (used for making squash preprations) for studying meiosis were fixed in Carnoy's fluid.

The customary methods of dehydration and imbedding were followed. Before microtoming, most of the imbedded material was soaked in water for about a month. Sections were cut 8-15 microns thick. A safranin-fast green combination was satisfactory but iron-haematoxylin proved to be better for female gametophytes containing archegonia. The extent of chromatin in the egg nucleus was determined by Feulgen nuclear reaction.

Acetocarmine preparations of free nuclear gametophyte and young embryos, dehydrated and mounted in canada balsam proved very useful. Microsporogenesis and the development of the pollen were studied mainly by squash technique.

External Morphology

Cephalotaxus drupacea grows as a small tree under cultivation at Dehra Dun. The leaves are arranged in a decussate and distichous fashion (Fig. 1A). They function for at least three years. The young branches are green and grooved while the old ones are brownish and smooth. Winter buds are covered by numerous scales (Fig. 1B). Some branches arise from the main stem near the soil level, grow vigorously and bear alternate leaves (Fig. 1C). Rarely the leaves may be decussate and four-ranked (Fig. 1D). Lateral buds on these branches lack the scales and unfold in the same year in which the shoot is produced (Fig. 1E). The branches bear decussate and two-ranked leaves only after two or three years of growth (Fig. 1F).

Winter buds in the female tree produce either (i) a leafy shoot, or (ii) a leafy shoot and two to four female cones (Fig. 1I), or (iii) only a few female cones (Fig. 1G). The buds of the first two categories have a more pointed apex than those of the third but there is no difference in their position. In the buds of the second type, after producing the female cones, the shoot apex gives rise to a foliage shoot while in the buds of the third category the shoot apex does not grow after producing the female cones. Since the resting buds mostly arise near the shoot apex, the female cones usually occur in that position (Fig. 1G). Occasionally the cones develop from the middle or the base of a branch (Fig. 1H).

Outwardly it is difficult to determine the point of origin of the female cones, because the bud scales from which they arise and the leaves[1] which subtend them look alike. However, a study of the formation of the lateral organs in the different seasons of a year clearly shows that the cones are borne in the axils of the lowermost two to four leaves on some newly formed shoots.

The female cones have a short stalk and are small in size. They are green at the time of emergence but acquire a reddish tinge as the resting period approaches, and consist of five to seven pairs of opposite and decussate bracts (Fig. 1J). The fleshy nature of the cone axis conceals the correct arrangement of bracts, but a study of the vascular anatomy makes this quite clear. Two ovules are borne in the axil of each bract (Fig. 1J) excepting the lowest pair. A small ridge-like outgrowth of the cone axis (Fig. 1L, N), generally

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1. The lateral organs in the axil of which cones are borne are not true leaves but are so termed because they are produced by the shoot apex during the period of foliage leaf formation (*see* Singh, 1961).
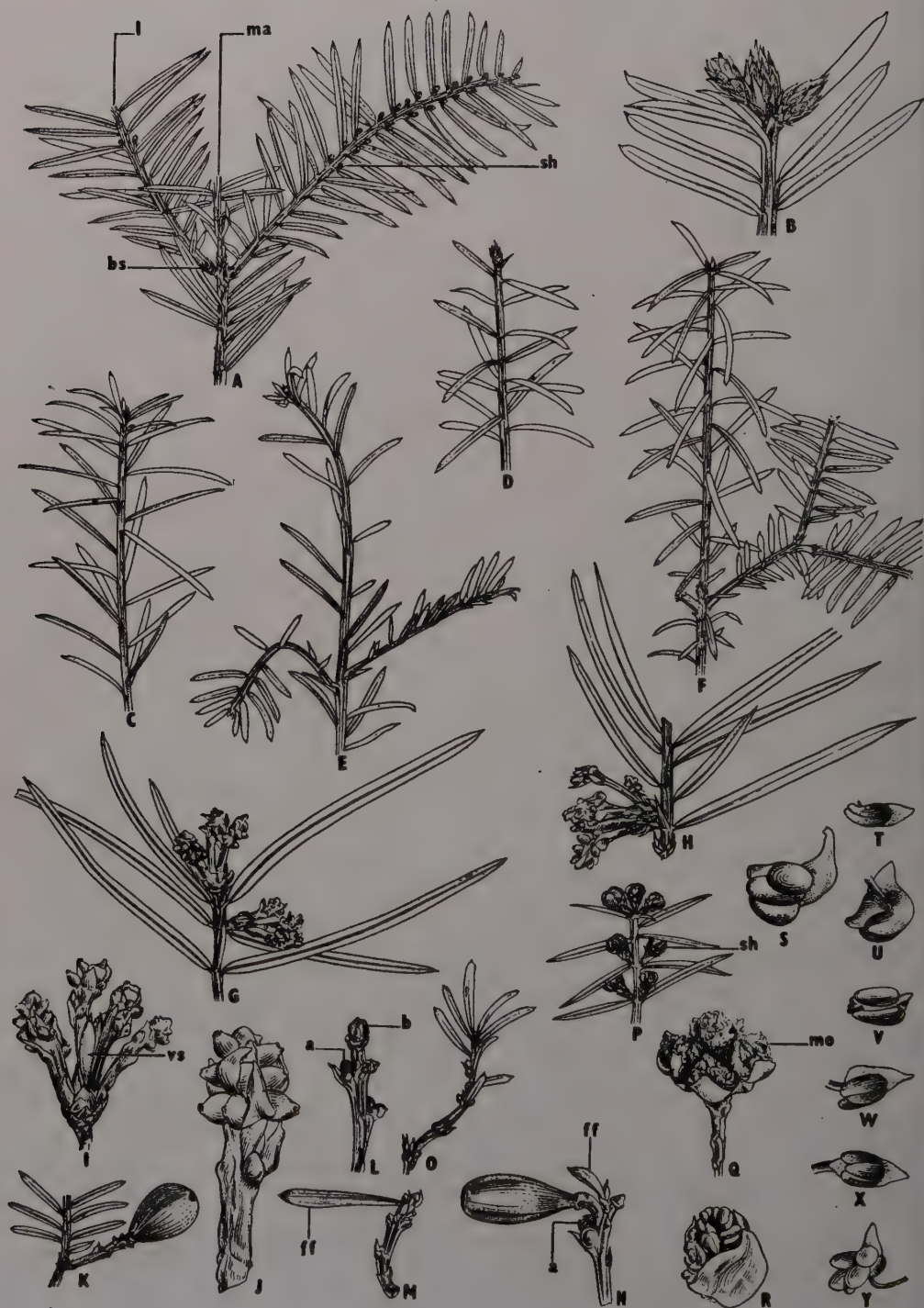
considered to be a secondary axis (Worsdell, 1901; Hirmer, 1936), is present between the two ovules of each bract. Florin (1938-45) has termed the two ovules and the outgrowth between them as a ' seed-scale complex '. The ovules that enlarge in the following spring are green and become red and fleshy on ripening. Generally one (Fig. 1K) or two ovules mature per cone although as many as five mature seeds have sometimes been observed.

Normally the apex of the female cones becomes inactive after producing the bracts and the ovules. However, a few cones were observed where the apex was surrounded by numerous bud scales (Fig. 1L). Favre-Duchartre (1957) has observed that the proliferation of such a bud may give rise to a female cone in the next year. In some cones the two ovules of a ' seed-scale complex ' were found to be replaced by two foliar leaves (Fig. 1M, N). The lower leaves of some foliar shoots were reduced and bore two ovules with the outgrowth between them (Fig. 1O). Such shoots were of a short length only. Worsdell (1901) has described several abnormal cones of *Cephalotaxus*. Abnormalities of the female cone have also been observed in many other conifers and at one time considerable significance was attached to them in interpreting the morphology of the cone.

Short and unbranched lateral shoots, which are closely covered with scales, arise in the axils of most of the leaves of the newly emerged shoot (Fig. 1A) of the male tree. Six to eight compactly arranged cones appear in the axils of the upper scales of these lateral shoots (Fig. 1P, Q). A male cone consists of a short sessile axis on which 15-20 microsporophylls are arranged spirally (Fig. 1R). The shape of the microsporophylls is variable (Fig. 1S-Y). A sporophyll consists of a stalk bearing three or four (rarely two, Fig. 1T) abaxial sporangia pointing toward the cone axis and a sterile flattened region which points outward and upward (Fig. 1S). Takhtajan's (1953) description of the microsporangia as ' pendant ' is erroneous although rarely a microsporophyll may be perisporangiate (*see* also Dluhosch, 1937).

Anatomy of Cones

MALE CONE — After the departure of the leaf trace (Fig. 2A, B), the part of the vascular cylinder lining each side of the leaf gap assumes a sickle-shape (Fig. 2C). These two portions become detached from the main cylinder (Fig. 2D) and fuse with each other to form a complete ring (Fig. 2E) which enters the axis of the short shoot bearing the male cones. Due to the departure of the traces to the sterile bracts borne on the lower portion of the lateral shoots, the vascular ring splits into several bundles (Fig. 2F). Each sterile bract is supplied by a single, unbranched bundle. Traces for the fertile bracts and the male cones depart at a

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FIG. 1A-Y — External morphology. (*a*, outgrowth between the two ovules; *b*, bud; *bs*, bud scales; *ff*, foliage leaf; *l*, lateral branch; *ma*, main axis; *mo*, male cone; *sh*, short lateral shoot which bears the male cones; *vs*, vegetative shoot). A. Young twig from male tree. Three branches have emerged from the previous year's shoot. The short lateral shoots situated in the axils of most of the leaves will bear the male cones. × 0·5. B. Branch bearing resting buds. × 2·2. C-F. Vigorously growing shoots which occur near the soil level. The leaves are alternate in C but opposite and decussate in D. E. Branches of the same year. F. Branches that have arisen after two or three years of growth. × 0·4. G, H. Female cones near the apex and base of two branches. ×2·2. I. Four female cones and a vegetative branch (*vs*) emerging from a resting bud. × 4·3. J. Female cone enlarged. × 8·7. K. Mature ovule on a female cone. × 0·7. L. A bud at the apex of a female cone. × 1·5. M, N. Female cones in which the two ovules of a ' seed scale complex ' have been replaced by foliage leaves. × 1·5. O. Short foliar shoot bearing ovules. × 0·7. P. Male cones borne on short lateral shoots are covered by scales. × 0·5. Q. A short lateral shoot enlarged. The male cones have become exposed. × 1·5. R. Male cone with the subtending bract. × 2·8. S. Typical microsporophyll. × 4·3. T-Y. Microsporophylls of various shapes. × 4·3.
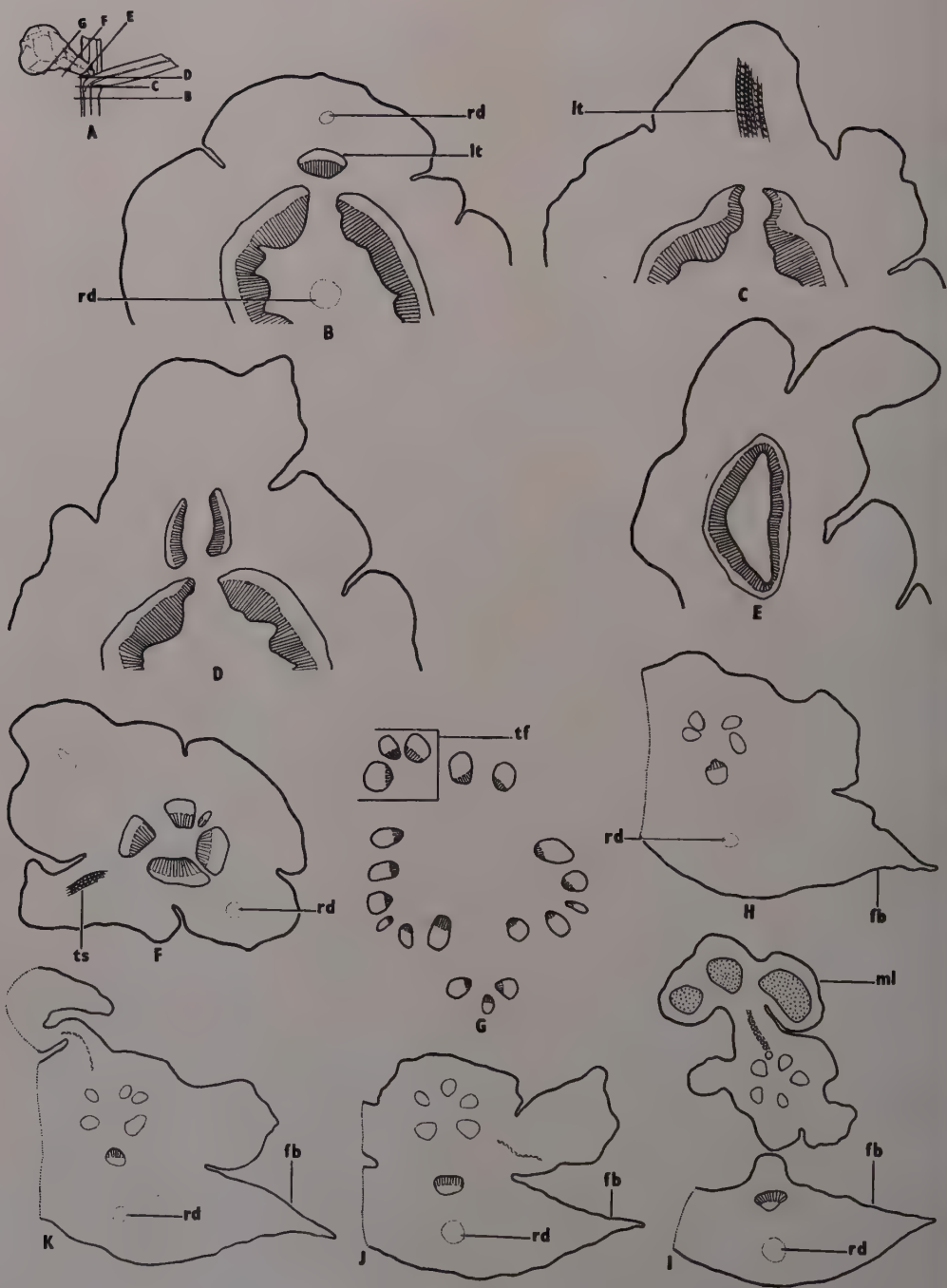
higher level. Three bundles (one small anterior and two large lateral) supply the bract and the axillary cone (Fig. 2G). The anterior bundle enters unbranched into the fertile bract, while the two laterals divide at a higher level to form four or five bundles arranged in a ring which enters the cone axis (Fig. 2H). One unbranched bundle is present in each sporophyll (Fig. 2I). A few sterile appendages having a vasculature similar to that of the sporophylls are sometimes present at the base of the cone (Fig. 2J, K).

FEMALE CONE — A single unbranched bundle supplies each bud scale (Fig. 3A, B). A little below the point where the female cone is attached to the main axis, a small anterior and two large lateral bundles become separated from the stele of the main axis (Fig. 3C). The anterior bundle supplies the reduced leaf (Fig. 3D, E) which bears the cone in its axil. The lateral bundles divide and form a ring which enters the axis of the female cone (Fig. 3D, E). The sterile bracts receive one bundle each while three bundles depart to supply the fertile bract with the axillary 'seed-scale complex' (Fig. 3F). The anterior bundle supplies the bract and is unbranched, while each of the lateral bundles bifurcates (Fig. 3G). The two daughter bundles orient themselves in such a way that the phloem of one faces that of the other (Fig. 3H, I). They run throughout the integument in this inverted fashion (Fig. 3J), each giving a horizontal branch in the middle of the integument, toward the inner part of the ovule (Fig. 3K). The branch consists of phloem elements only. They show abundant centripetal xylem.

Microsporangium

A plate of hypodermal archesporial cells, five to seven cells wide and four or five cells long, is present on the abaxial surface near the base of the microsporophyll (Fig. 4A-C). Later, due to sterilization at places, two to four groups of archesporial cells are delimited (Fig. 4D) each becoming the seat of a microsporangium. The archesporial cells divide periclinally to form the primary parietal layer, which organizes a four-layered wall, and the primary sporogenous layer (Fig. 4D). The latter divides several times producing a mass of sporogenous cells (Fig. 4E). The upper portion of the sporophyll forms the sterile flattened region. Its cells take a uniformly dense stain and occasionally show a resin duct (Fig. 4F, G). The microsporangia grow toward the axis of the cone (Fig. 4F, G). They are free from each other at their tips but are united at the base (Fig. 4H-J).

Some cells of the wall layers contain darkly staining substances (*pb*) and a few others show tannin (Fig. 4K). The cells of the innermost wall layer undergo frequent anticlinal divisions and differentiate into tapetum (Fig. 4K). Some of its cells become binucleate at the mother cell stage of the sporangium (Fig. 4L). The tapetum is of the glandular type and degenerates simultaneously with the reduction divisions in the mother cells (Fig. 4M). The epidermal cells become highly cutinized, their radial and inner

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FIG. 2A-K — Anatomy of male cone. (*fb*, fertile bract; *lt*, leaf trace; *ml*, microsporophyll; *rd*, resin duct; *tf*, traces to the fertile bract and the male cone; *ts*, trace to the sterile bract). A. A portion of stem bearing the short lateral shoot which gives rise to the male cones. The levels of the transverse sections represented in B-G are marked B-G. × 2. B-D. Transections of stem showing the departure of the leaf trace and the origin of the vascular supply for the lateral shoot. × 35. E. Section at the base of the lateral shoot. × 35. F. Same at a higher level to show the traces to the sterile bracts. × 35. G. Departure of three bundles to the fertile bract and the male cone. × 35. H-K. Transections at different levels of a male cone to show the departure of traces to the microsporphyll (I) and the sterile appendages that are occasionally present at the base of the male cone (J, K). × 35.
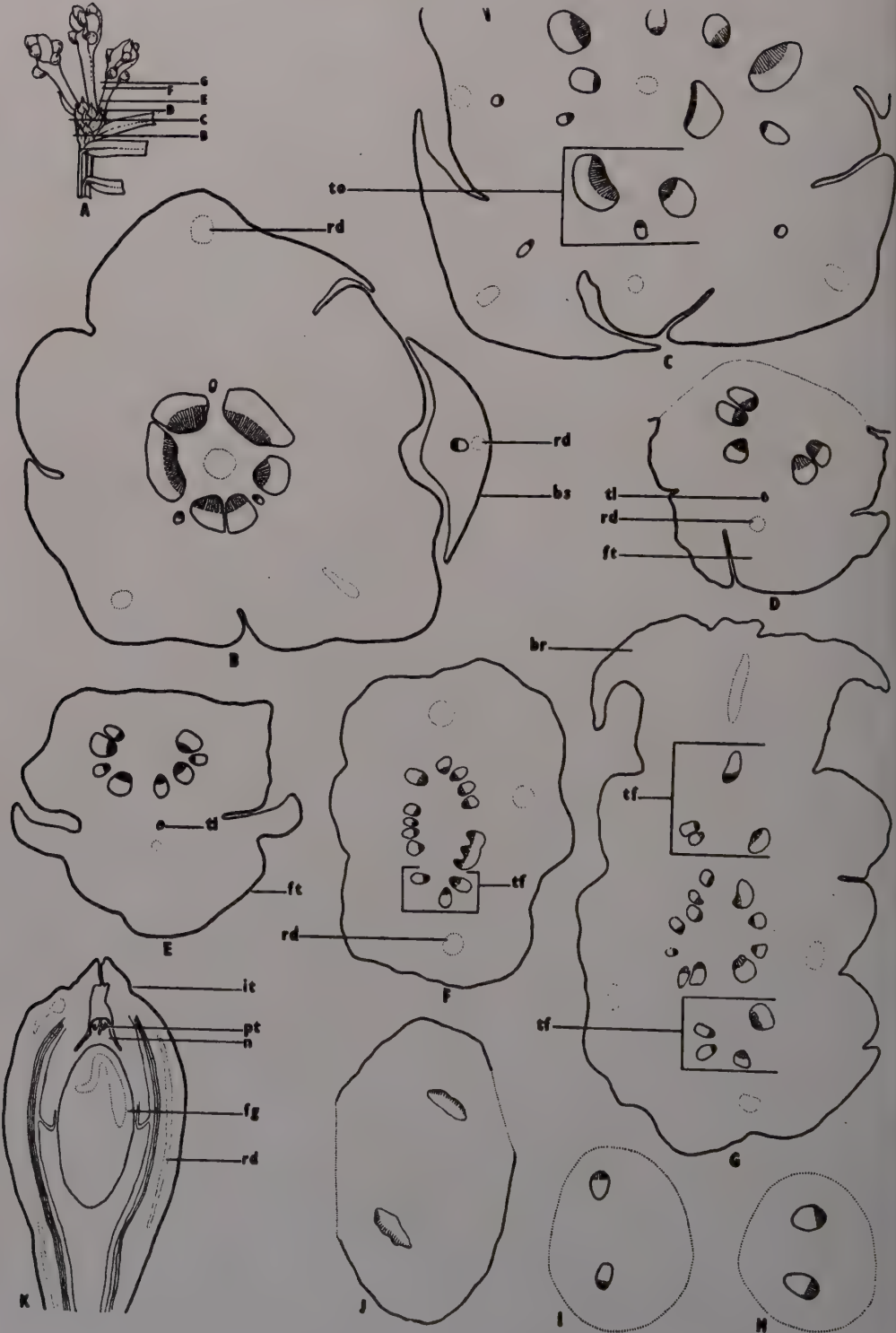
tangential walls become thick, and develop fibrous thickenings as the microspores undergo their first division. At maturity the epidermis alone forms the wall of the sporangium (Fig. 4N).

A few cells of the epidermis of each sporangium facing the stalk of the microsporophyll do not develop any thickenings (Fig. 4O). Dehiscence occurs along these unthickened cells (Fig. 4P). The partition walls between the sporangia, where they are united to each other, collapse (Fig. 4Q).

Microsporogenesis

The sporogenous cells divide to form the microspore mother cells which are full of starch grains (Fig. 5A). In preparation for meiosis, their cytoplasm contracts from the wall and a special mucilaginous sheath is secreted around the cytoplasm (Fig. 5A). At diakinesis 12 bivalents were counted (Fig. 5B). This figure tallies with all the previous reports on chromosome number of the genus (Sax & Sax, 1933; Sugihara, 1940; Mehra & Khoshoo, 1956). Wall formation takes place only after Meiosis II (Fig. 5C-E). It was observed that while some of the mother cells in a sporangium are in Metaphase I, the others might have completed Telophase II. The number of starch grains decreases considerably after the completion of meiosis. The microspore tetrads may be tetrahedral or isobilateral (Fig. 5F, G).

Several abnormalities were observed during microsporogenesis. Occasionally some chromosomes do not arrange themselves along the plate during Metaphase I (Fig. 5H). These chromosomes form micronuclei. One or both the nuclei may not undergo Meiosis II resulting in a triad or two ' microspores ' (Fig. 5I-K). Rarely a mother cell becomes transformed directly into a microspore (Fig. 5L). Such pollen grains can be recognized by their large size. Frequently wall formation in tetrads is incomplete resulting in two- or three-nucleate microspores, with or without a constriction between the nuclei (Fig. 5M-P). The division of the nuclei in the compound spore may or may not take place simultaneously (Fig. 5Q-S). Polysporic ' tetrads ' (Fig. 5T) are not uncommon and up to eight microspores have been seen in a single group. Rarely polyspory and the non-separation of spores may go together to produce an abnormal clump of spores (Fig. 5U). At times the microspores do not develop the exine even after separating from each other (Fig. 5J, K). Khoshoo (1957a, b) has observed ' tetrads ' containing one to ten microspores and attributes this feature to a partial failure of spindle formation during meiosis leading to irregular distribution of chromosomes and polyspory, and suppression of Meiosis II resulting in dyads or triads.

Male Gametophyte

Soon after formation, the microspores are released from tetrads by the breaking down of the mucilaginous sheath and the original wall of the mother cell. When released, the spores are small and round with a centrally situated nucleus (Fig.

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Fig. 3A-K — Anatomy of female cone. (*br*, bract; *bs*, bud scale; *fg*, female gametophyte; *ft*, fertile leaf; *it*, integument; *n*, nucellus; *pt*, pollen tube; *rd*, resin duct; *tf*, traces to the fertile bract and the ' seed-scale complex '; *tl*, trace to the fertile leaf; *to*, traces to the female cone and the fertile leaf). A. Apex of a branch bearing three female cones. The levels of the transections are marked B-G. × 2. B. T.s. bud showing the vascular traces for the bud scales. × 31. C. Same at a higher level showing traces to the fertile leaf and the female cone. × 31. D, E. Transections at the base of the female cone. The fertile leaf (*ft*) is not yet separated. × 31. F, G. Sections of female cone showing the origin of the traces (*tf*) for the fertile bract and the 'seed-scale complex'. Some of the ovular bundles have bifurcated in G. × 31. H, I. Change in orientation of the two vascular bundles entering an ovule, the phloem of the bundles faces each other. × 31. J. T.s. ovule at the base to show inverted vascular bundles. × 31. K. L.s. ovule showing horizontal strands given out from the vascular bundles. × 8.
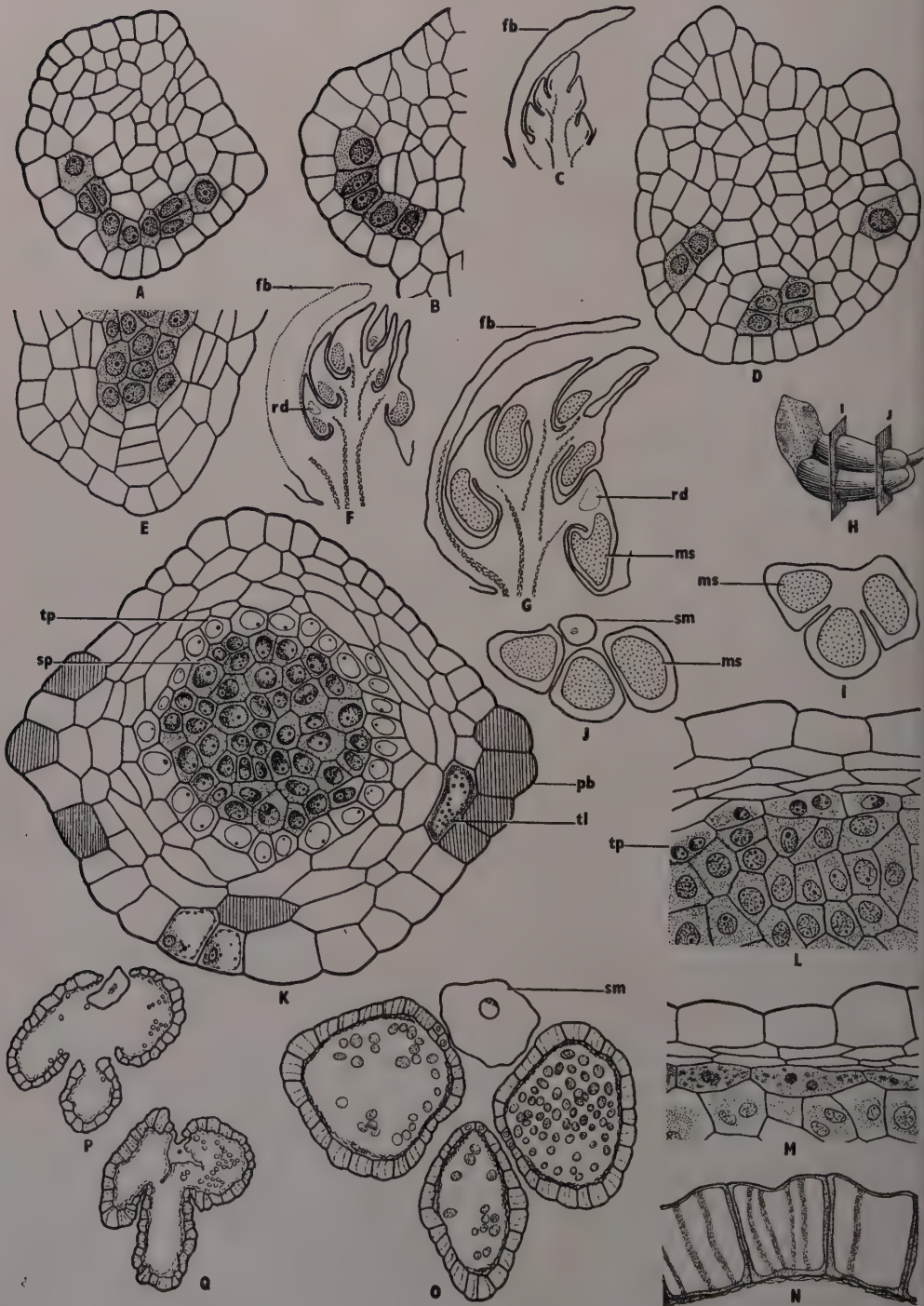
5V). Their cytoplasm contains a few starch grains. The intine soon becomes thick, but the exine remains thin (Fig. 5W). The nucleus divides to form a lenticular antheridial cell and a large tube cell (Fig. 5X, Y). Prothallial cells are absent. The pollen grain enlarges about twice its original size, after which the exine invariably ruptures and the intine appears thinner perhaps due to expansion (Fig. 5Z). Starch grains disappear and the pollen grains are shed at this stage.

The pollen grains are caught in the pollination drop exuded from the micropyle and are ' sucked in '. The contents along with the sheathing intine escape from the cracked exine if the pollen grains are floated either in water or a weak solution of sucrose. Since the exine rarely reaches the nucellus, it is probable that the ruptured exine is cast off when the pollen grain is caught in the pollination drop.

The pollen settles on the degenerated top of the nucellus (a weakly developed pollen chamber). The intine enlarges at the tube cell end to form a short but broad pollen tube which penetrates the nucellus (Fig. 6A, E). During its growth the pollen tube crushes the nucellar cells. According to Favre-Duchartre (1957) the pollen tube grows between the nucellar cells by marginal outgrowths. However, present observations do not confirm this. Favre-Duchartre (1957) counted as many as 12 pollen tubes in the same nucellus but I have never seen more than four.

The tube nucleus migrates into the pollen tube. The antheridial cell enlarges considerably and divides at right angle to the long axis of the pollen tube, to form the stalk cell and the body cell (Fig. 6B). The stalk cell soon gets devoid of a clear wall (Fig. 6C). Both the cells move down and lie free in the cytoplasm of the tube (Fig. 6D). Up to this time the stalk nucleus is surrounded by dense cytoplasm (Fig. 6D) but a little after the stalk and the body cells reach near the tube nucleus, the cytoplasm around the stalk nucleus becomes indistinguishable (Fig. 6E, F). From now onwards the pollen tube thus contains a body cell, a stalk nucleus and a tube nucleus — the latter two being similar to each other. During the growth of the pollen tube, the stalk and the tube nuclei usually precede the body cell which remains close to them (Fig. 6G-M). The contents of the pollen tube lie at its tip. However, Favre-Duchartre (1957) has observed some pollen tubes in which they may lie far behind the tip. The tube retains this stage for about one year, the only change being that the body cell enlarges somewhat (Fig. 6G).

In the following year the pollen tube grows faster. When it reaches the female gametophyte, the archegonia are almost mature (Fig. 6H). The tube enters the archegonial cavity, comes in contact with the neck cells of the archegonium and becomes swollen at the tip (Fig. 6H, I). By this time the body cell has enlarged considerably. Its nucleus, which is now very prominent, divides but no wall is laid down between the two male nuclei (Fig. 6J). To begin with they are small,

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Fig. 4A-Q — Microsporangium. (*fb*, fertile bract; *ms*, microsporangium; *pb*, cells having darkly staining contents; *rd*, resin duct; *sm*, stalk of the microsporophyll; *sp*, sporogenous tissue; *tl*, tannin cell; *tp*, tapetum). A, B. Cross and longisections of young sporophylls showing the extent of the hypodermal archesporium. × 315. C. L.s. young male cone. × 30. D. Transection of a sporophyll. Three sporangia have been demarcated. × 315. E. Differentiation of wall layers and the division of the sporogenous cells. × 315. F, G. Longisections of male cones in different stages of development. × 30. H. A microsporophyll showing levels of sections represented in I, J. × 8. I, J. Sections of sporophylls at levels indicated above. × 30. K. Cross-section of a microsporangium; the tapetum has differentiated. × 315. L. A portion of a sporangium during the resting stage. Some of the tapetal cells are binucleate. × 315. M. Wall layers of the sporangium at reduction divisions. × 315. N. Same at the shedding stage. × 315. O. Cross-section of a mature sporophyll. Note the unthickened cells of the walls of the sporangia. × 56. P, Q. Sections of a sporophyll at different levels, showing dehisced sporangia. × 30.
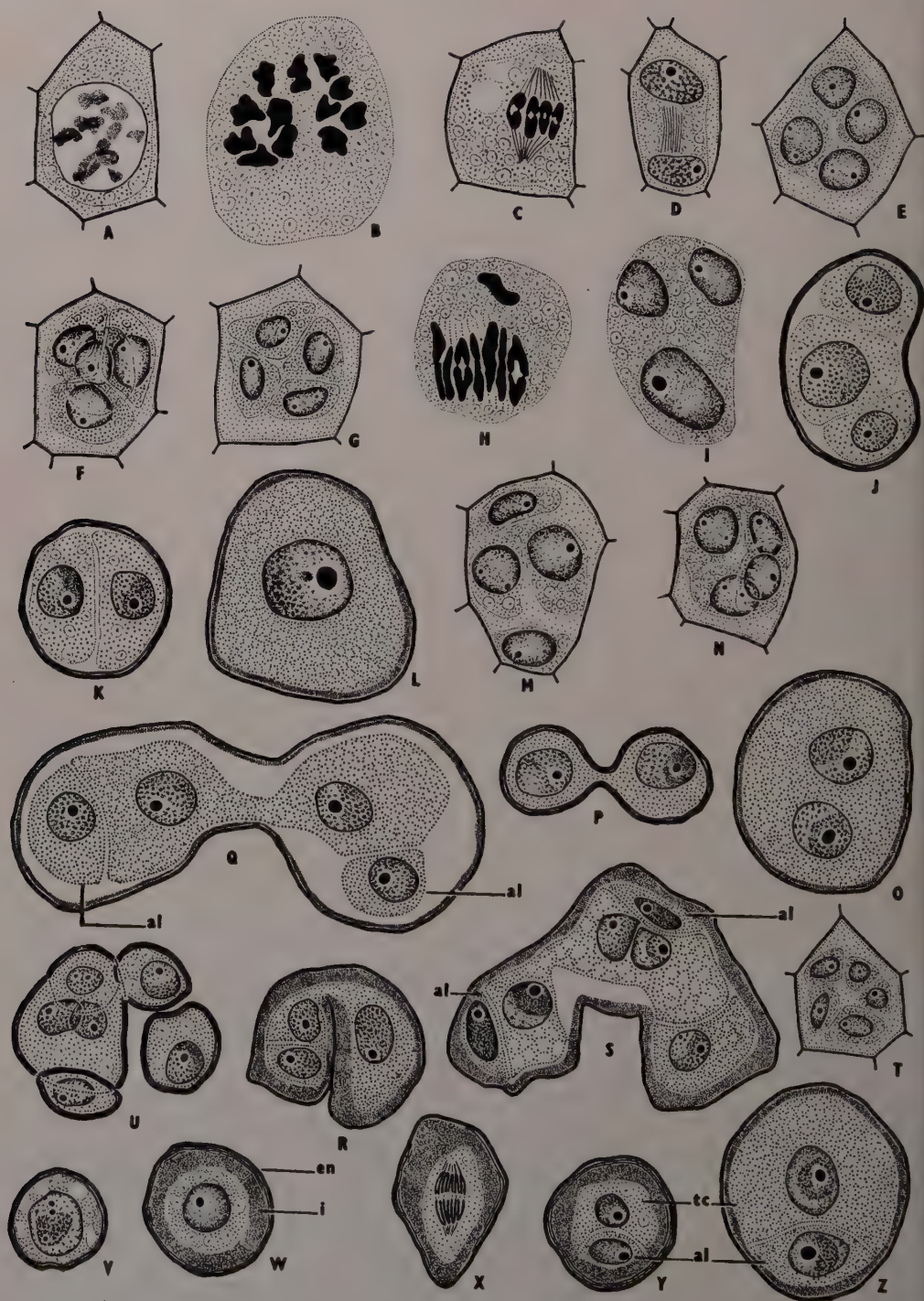
and have dense chromatin and a prominent nucleolus (Fig. 6 J, K). At maturity the male nuclei become larger and are mostly filled with nucleoplasm. The nucleolus disappears and the chromatin is not sharp (Fig. 6L, M). At this time the cytoplasm of the body cell and the pollen tube contains small, rounded, densely staining bodies (Fig. 6L, M). The male nuclei are unequal in *C. drupacea* var. *pedunculata* (Kaur, 1958).

The neck cells begin to degenerate as the pollen tube comes in contact with them (Fig. 6 I), and a passage is formed for the tube to enter the egg. The male nuclei separate from each other due to the breaking of the wall of the body cell (Fig. 6M). The stalk and the tube nuclei degenerate (Fig. 6M). The tip of the pollen tube comes to lie inside the archegonium, where it bursts and one or both the male nuclei together with some cytoplasm of the body cell enter the egg cell.

Megasporangium

An outgrowth arises in the axil of every fertile bract of a young female cone (Fig. 7A, B). Its apex, which shows but little growth, soon acquires the typical organization of a vegetative shoot apex, i.e. the four usual cytohistological zones (Singh, 1961) can be demarcated (Fig. 7C). Thus the outgrowth apparently represents a secondary axis. The two ovules arise as lateral protuberances from this secondary axis and in younger stages grow mainly by periclinal divisions (Fig. 7D) which characterise a leaf primordium.

The next available stage showed a well formed integument and three to six hypodermal archesporial cells which were not always in a single layer (Figs. 8A, 11A). The archesporial cells divide periclinally to form the sporogenous layer and the primary parietal layer (Fig. 8B). The latter undergoes repeated periclinal divisions to form the massive nucellus (Fig. 8C, D). The nucellar cells in the vicinity of the sporogenous layer at the chalazal end also undergo periclinal divisions (Fig. 8E) to form a distinct tissue (here designated as pavement tissue) which in later stages contains large compound starch grains (Figs. 8F, 11E). The tissue gets crushed during the enlargement of the free nuclear gametophyte.

A few days before pollination, two or three layers of nucellar cells at the micropylar region start degenerating and form a rudimentary pollen chamber (Fig. 9A-C). Side by side a band of cells of the inner epidermis of the integument lining the micropyle becomes active (Fig. 9B). After pollination these cells grow inward, undergo a few transverse divisions and close the micropylar opening (Fig. 9D-F), thus effectively sealing the freshly arrived pollen. As the ovules mature these elongated cells acquire a very thick wall. Some epidermal cells at the junction of the nucellus and the integument accumulate tannin (Fig. 9G). Figure 9H shows the condition of the ovule during the second winter.

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Fig. 5A-Z — Microsporogenesis and male gametophyte. (*al*, antheridial cell; *en*, exine; *i*, intine; *tc*, tube cell.) A, C-G, M, N, R-Z are from microtome sections while the rest are from whole mounts. A-D. Stages in Meiosis I of the microspore mother cell. × 789. E. Cytokinesis. × 789. F, G. Tetrahedral and isobilateral tetrads. × 789. H. Disturbed Metaphase I. × 789. I, J. Only one of the dyad cells has undergone Meiosis II. × 789. K. Formation of exine at dyad cell stage. × 789. L. Pollen grain derived from mother cell without undergoing meiosis. × 789. M, N. Tetrads showing incomplete separation of microspores. × 789. O-S. Incompletely separated microspores. The two nuclei in the spore have behaved similarly in Q; in R the division has taken place only on the left side. In S only two nuclei out of the four have divided. × 789. T. A polysporic tetrad. × 789. U. A tetrad which has probably arisen by polyspory and the incomplete separation of spores. × 789. V, W. Uninucleate microspores. × 789. X. Microspore nucleus dividing. × 789. Y, Z. Two-nucleate pollen grains. × 789.
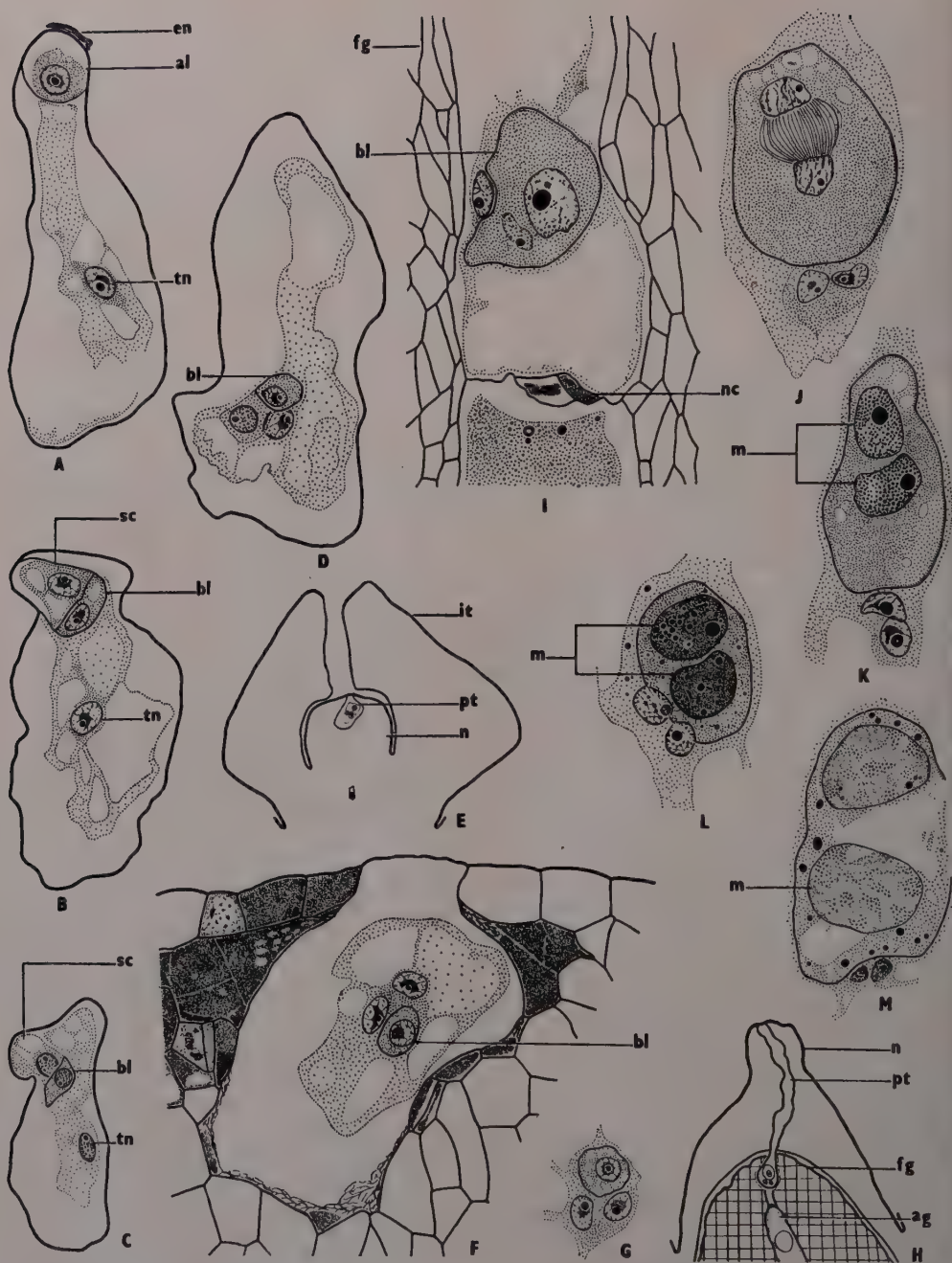
Fig. 6A-M — Male gametophyte. (*ag*, archegonium; *al*, antheridial cell; *bl*, body cell; *en*, exine; *fg*, female gametophyte; *it*, integument; *m*, male nuclei; *n*, nucellus; *nc*, neck cells; *pt*, pollen tube; *sc*, stalk cell; *tn*, tube nucleus). A. Two-celled pollen tube. The exine of the pollen grain can be seen above the antheridial cell. × 317. B. The antheridial cell has divided. × 317. C. The wall around the stalk cell is not distinct. × 317. D. The stalk and the body cells have reached near the tube nucleus. The stalk nucleus has dense cytoplasm around it. × 317. E. L.s. ovule showing a pollen tube in the nucellus. × 31. F. Pollen tube enlarged from E. × 317. G. The nuclei of a pollen tube from a collection one year later than that in F. × 317. H. L.s. upper part of ovule showing nucellus (*n*), pollen tube (*pt*) and archegonium (*ag*). × 31. I. Tip of the pollen tube just above an archegonium. × 317. J. Division of body cell. × 317. K-M. Maturation of male nuclei; the stalk and the tube nuclei have degenerated in M. × 317.
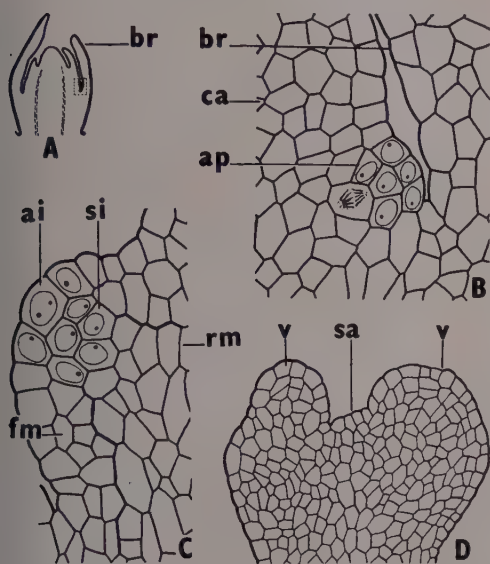
FIG . 7A-D — Megasporangium. (*ai*, apical initials; *ap*, primordium of the secondary axis; *br*, bract; *ca*, cone axis; *fm*, flank meristem; *rm*, rib meristem; *sa*, secondary axis; *v*, ovular primordium). A. L.s. very young female cone. × 32. B. The portion marked in A enlarged to show the initiation of the secondary axis. × 334. C. A later stage in the development of the secondary axis. The tip shows the typical organization characteristic of the shoot apex. × 334. D. Initiation of the two ovules from the secondary axis. × 143.

Megasporogenesis

One of the sporogenous cells enlarges considerably especially along its longer axis and acquires a large nucleus and frothy cytoplasm (Figs. 10A, B, 11C). This cell functions as the megaspore mother cell (Fig. 10C). The non-functional sporogenous cells can be recognized in the vicinity of the mother cell. Meiosis I results in the formation of two dyad cells. Meiosis II usually precedes in the chalazal dyad cell than in the micropylar one (Fig. 10D). Linear tetrads are formed and the chalazal megaspore functions (Fig. 10E, F). There is no regularity in the order of the degeneration of the three micropylar spores (Fig. 10E, G). A few cases were observed of an intermediate megaspore enlarging and the other three degenerating (Fig. 10H).

The functional megaspore becomes vacuolate (Fig. 11D) and its nucleus divides to form an 8- or 16-nucleate, somewhat elongated female gametophyte (Fig. 12A). It continues to enlarge by consuming the surrounding nucellar cells (Fig. 12A). The free nuclei become arranged in a single layer along the periphery leaving a central vacuole. At the 32-nucleate stage the gametophyte has a two-nucleate (rarely four-nucleate) finger-like projection at the micropylar end (Fig. 12B, C). This projection remains prominent until about 128 nuclei have been formed (Fig. 12D), but later it becomes levelled and is no longer conspicuous (Fig. 12E). A similar micropylar projection of the free nuclear gametophyte has also been figured in some other conifers. In *Taxus* (Dupler, 1917) it is enucleate, while in *Torreya* (Coulter & Land, 1905), *Austrotaxus* (Saxton, 1934) and *Saxegothaea* (Looby & Doyle, 1939) it contains one or two nuclei.

In later stages the gametophyte becomes oblong (Fig. 12E, G) and shows a thin membrane. Favre-Duchartre (1957) and Sokolowa (1890) have also observed a similar membrane. Wall formation is initiated after several hundred nuclei are formed (4096 according to Favre-Duchartre, 1957) and takes place by centripetally advancing cells which show a large central vacuole with the nucleus either at the periphery of the gametophyte or the inner end (Fig. 12H). These cells divide even before they reach the centre of the gametophyte (Fig. 12H). As the gametophyte advances in age, its nuclei show a gradual reduction in size (Fig. 12B-H). Its cells are arranged in files converging toward the centre. They contain scanty cytoplasm and have thin walls. Binucleate cells are also common. As the embryo matures the cells of the gametophyte become densely cytoplasmic and are finally filled with starch grains and oil globules.

After the central vacuole of the gametophyte is completely filled with cells, two to five surface cells at the micropylar end enlarge, acquire dense cytoplasm and large nuclei (Fig. 12I), and constitute the archegonial initials. The cells in their
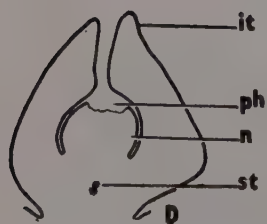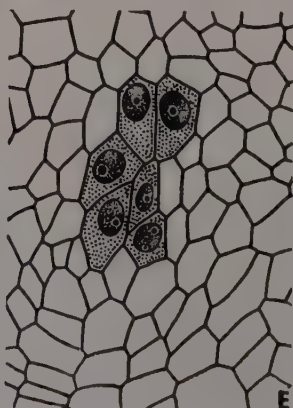
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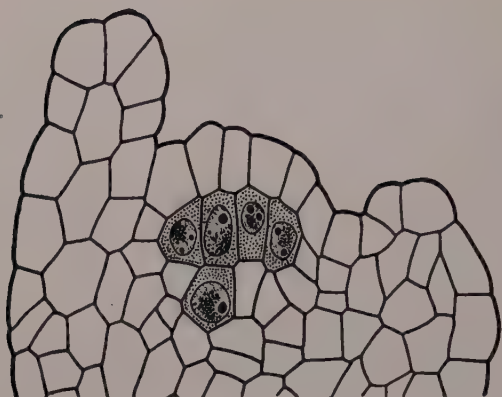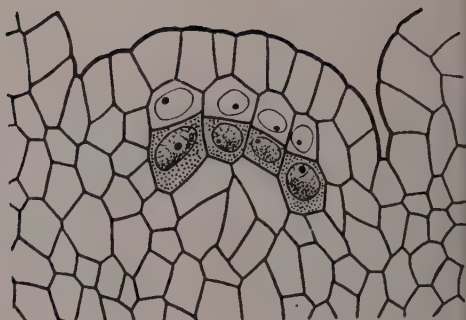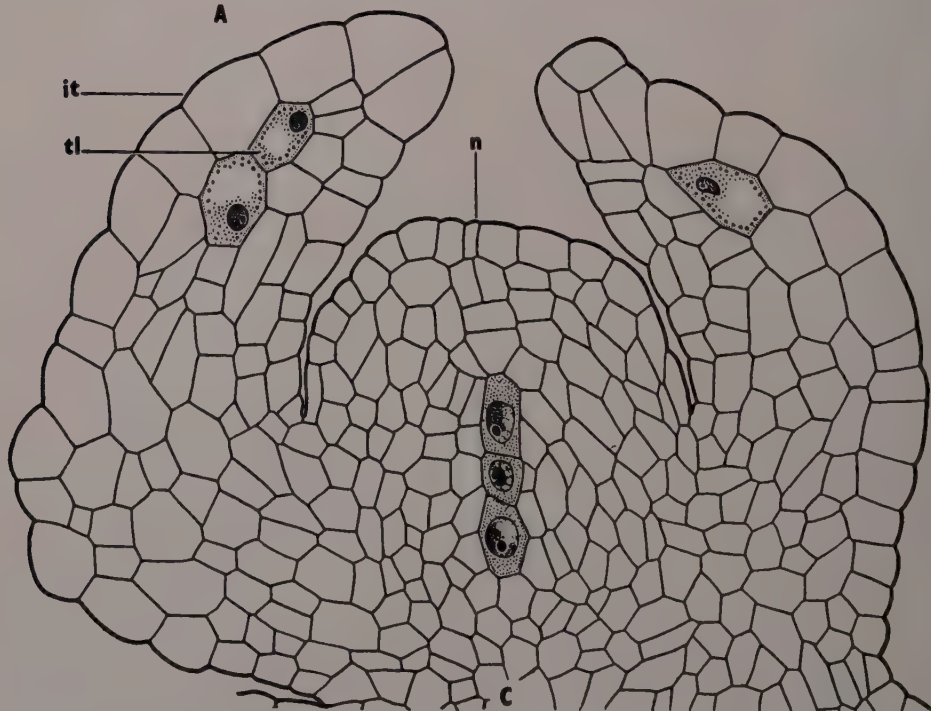
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F

immediate vicinity undergo many periclinal divisions and differentiate into the jacket layer. Its cells are isodiametric to begin with but later become elongated. According to Favre-Duchartre (1957) the jacket cells frequently contain two nuclei, but in my material such a condition was rather rare.

The archegonial initials divide periclinally to form the large central cell and the small neck initial (Fig. 12J). The latter divides anticlinally to form a two-celled neck composed of one tier (Fig. 12M, N). Sometimes three or four neck cells are formed and rarely the neck may consist of two tiers of cells (Fig. 12K, L). The outer tangential walls of the neck cell are always thick (Fig. 12K-O). During the foam stage of the archegonium the cells of the gametophyte at the micropylar end elongate upward and divide periclinally (Fig. 12M-O). Favre-Duchartre (1957) failed to observe any periclinal divisions in them. As the archegonium matures, these cells become richly cytoplasmic and frequently show two nuclei. They[2] are specially prominent during the development of the embryo (Fig. 17A) and rarely form a specially raised protuberance[3] (Fig. 12P, Q). The neck cells appear sunken so that each archegonium comes to have its own archegonial chamber (Fig. 12 O, Q).

The nucleus of the central cell is densely chromatic, has a prominent nucleolus and lies just below the neck cells (Figs. 12M, N; 13A). The central cell elongates considerably and has a pointed chalazal

end. Its cytoplasm becomes vacuolated (foam stage of the archegonium) and contains many irregular and darkly staining bodies.

After the central cell has reached its full size, the vacuoles begin to disappear, the cytoplasm becomes dense and round protein granules make their appearance (Fig. 13B). Its nucleus divides to form the egg and the ventral canal nuclei which are equal in size and not separated by a wall (Fig. 13B, C). However, the ventral canal nucleus is ephemeral and is soon reduced to a faintly staining body (Fig. 13D), which completely disappears later (Fig. 13E). The egg nucleus migrates downward and enlarges considerably (Fig. 13E). Meanwhile minute darkly staining bodies appear along the inner surface of the nuclear membrane, the nucleolus disappears and the chromatin becomes indistinct (Fig. 13D, E). The mature egg nucleus, stained with Schiff's reagent, revealed that the chromatin is restricted to a small dot which may be present anywhere in the nucleus. At the time of fertilization the egg nucleus is quite large, full of nucleoplasm and contains many darkly staining rounded bodies which are unlike nucleoli.

The mature archegonium is long, narrow and pointed at the chalazal end (Fig. 13F). It contains dense cytoplasm and numerous protein globules (Fig. 13E). The archegonia are situated a little below the tip of the gametophyte and are separated from each other by four to eight layers of cells. Occasionally archegonia are seen in a somewhat lateral position (Fig. 13G).

Frequently the nucleus of the central cell lies much below the neck cells or in the middle (Fig. 13H, I). Such nuclei do not divide but undergo the enlargement phase (during which minute round bodies appear

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2. A large cone of similar cells is also present in some members of the Podocarpaceae (Looby & Doyle, 1939, 1944; Boyle & Doyle, 1953).

3. Sahni (1921) termed this specially raised protuberance as a 'tent pole' but Favre-Duchartre (1957) does not agree with this view.

FIG. 8A-F — Megasporangium. (*it*, integument; *n*, nucellus; *ph*, pollen chamber; *st*, sporogenous tissue; *tl*, tannin cell). A. L.s. ovule showing hypodermal archesporium. × 395. B. The archesporial cells have divided periclinally to form the primary parietal layer and the sporogenous layer. × 395. C. L.s. ovule in the resting stage during the first winter. Note the tannin cells in the integument. × 395. D. Ovule at the time of pollination. × 38. E. Sporogenous tissue enlarged. × 395. F. Pavement tissue in late stage of development. × 395.
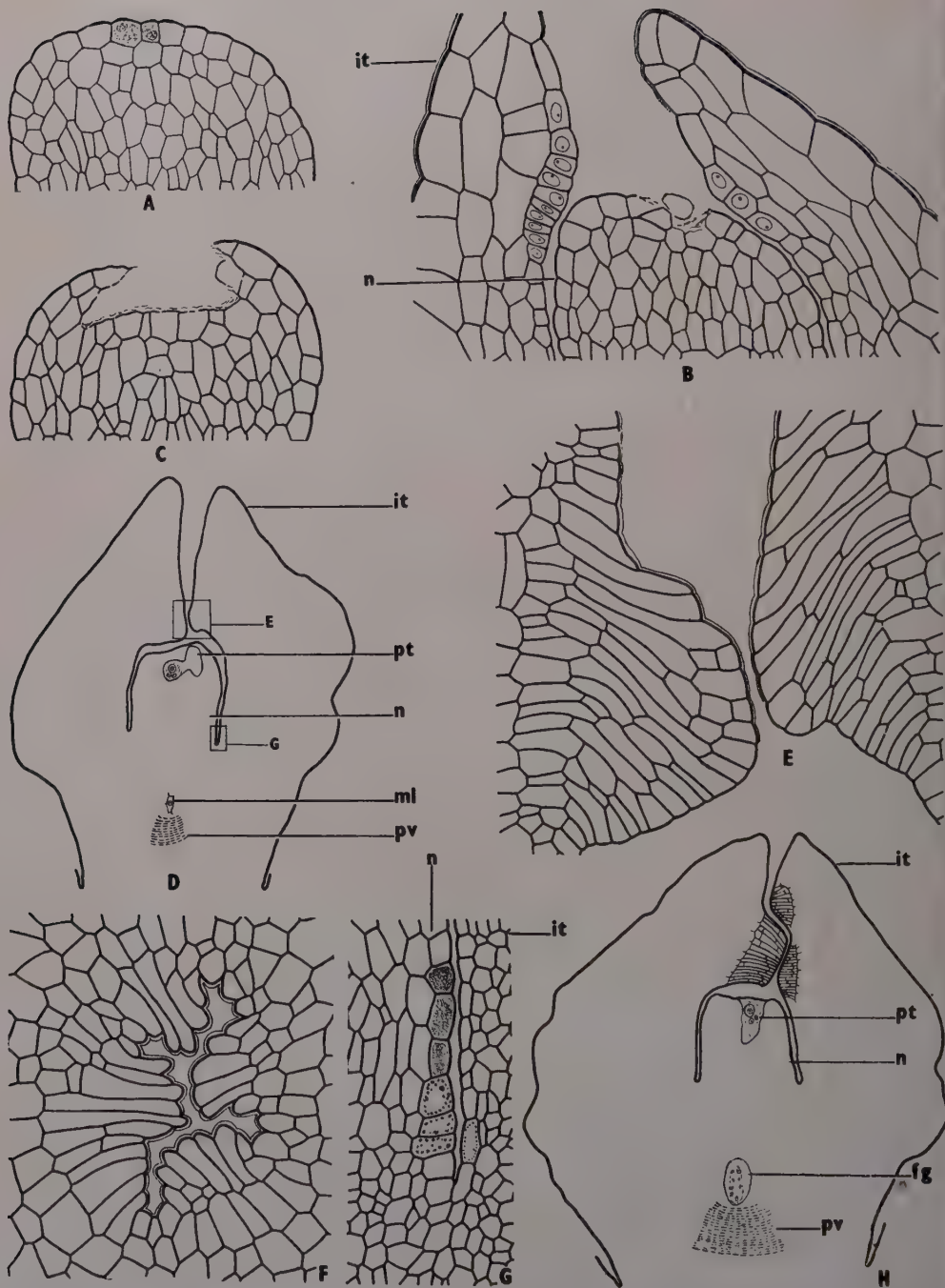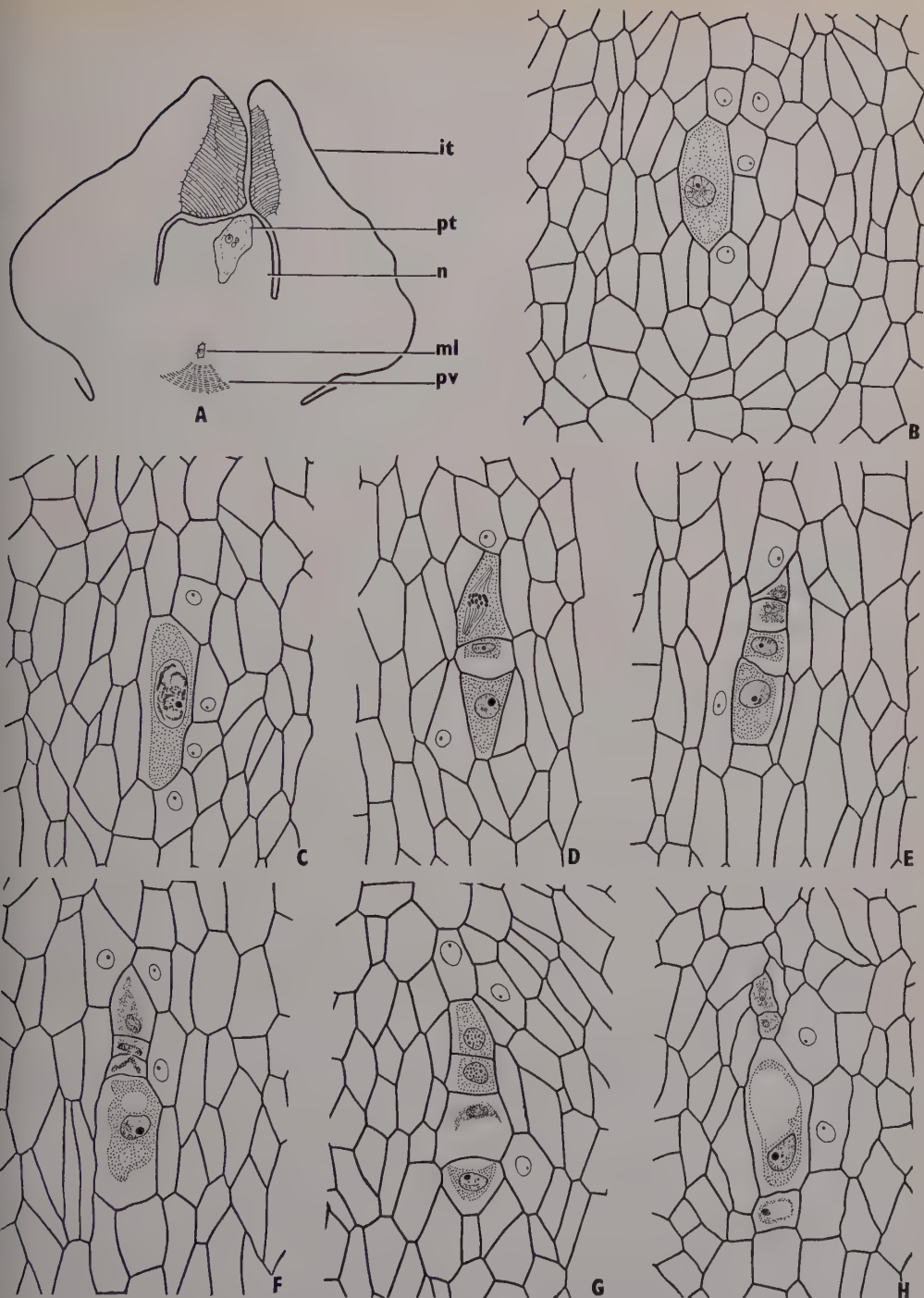
FIG. 9A-H — Megasporangium. (*fg*, female gametophyte; *it*, integument; *ml*, megaspore mother cell; *n*, nucellus; *pt*, pollen tube; *pv*, pavement tissue). A-C. L.s. upper part of nucellus to show the formation of the rudimentary pollen chamber. In B note the band of active inner epidermal cells of integument. × **168**. D. Outline diagram for E, G. × **37**. E. Portion marked E in D enlarged to show the closure of micropyle. × **168**. F. T.s. closed micropyle. × **168**. G. Portion marked G in D enlarged. Note the epidermal cells of nucellus and integument containing tannin. × **168**. H. L.s. ovule collected in the second winter. × **37**.

Fig. 10A-H — Megasporogenesis. (*it*, integument; *ml*, megaspore mother cell; *n*, nucellus; *pt*, pollen tube; *pv*, pavement tissue). A. L.s. ovule at the time of differentiation of the megaspore mother cell. × 38. B. Mother cell from A enlarged. Some of the non-functional sporogenous cells can also be seen. × 370. C, D. Stages in meiosis. × 370. E. A linear tetrad; the two micropylar megaspores have already degenerated. × 370. F-H. Degeneration of non-functioning megaspores; note lack of any definite sequence in degeneration. × 370.
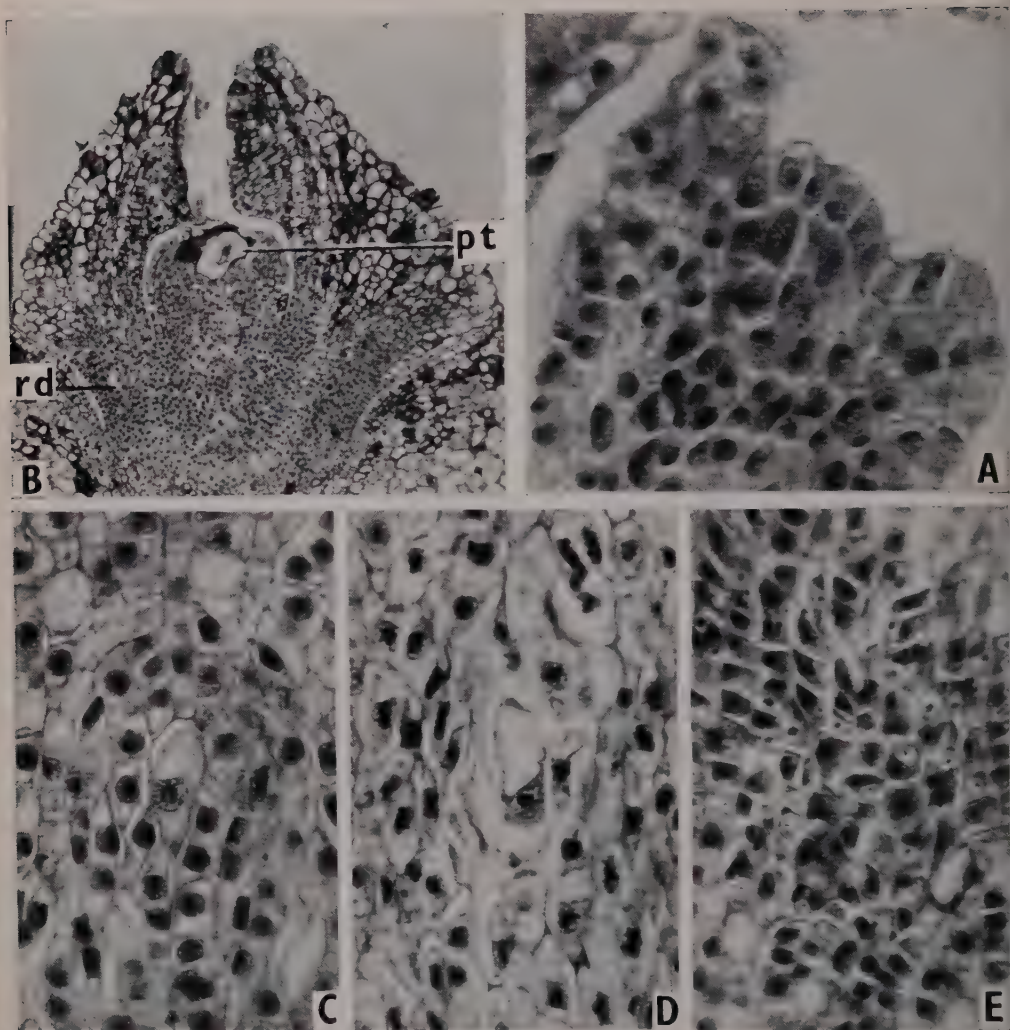
FIG. 11A-E — Megasporangium and megasporogenesis. (*pt*, pollen tube; *rd*, resin duct). A. L.s. very young ovule showing hypodermal archesporium. × 235. B. L.s. ovule collected one month after pollination; a resin duct has started developing in the integument. × 56. C. Megaspore mother cell enlarged from B. × 178. D. Functional megaspore. × 237. E. Pavement tissue. × 235.

along the inner periphery of the nuclear membrane) typical of the egg nucleus and occupy a central position (Fig. 13J). Rarely the nucleus may even enlarge while in its original place near the neck cells (Fig. 13K), and then migrate downward and function as the egg nucleus. At maturity such archegonia cannot be differentiated from the normal archegonia.

However, the ventral canal nucleus is absent in both these types. Very rarely an egg contains two large nuclei (Fig. 13L). According to Favre-Duchartre (1957) these arise by a division of the egg nucleus.

As already stated the pollen tube reaches the female gametophyte when the archegonia are almost mature. Rarely
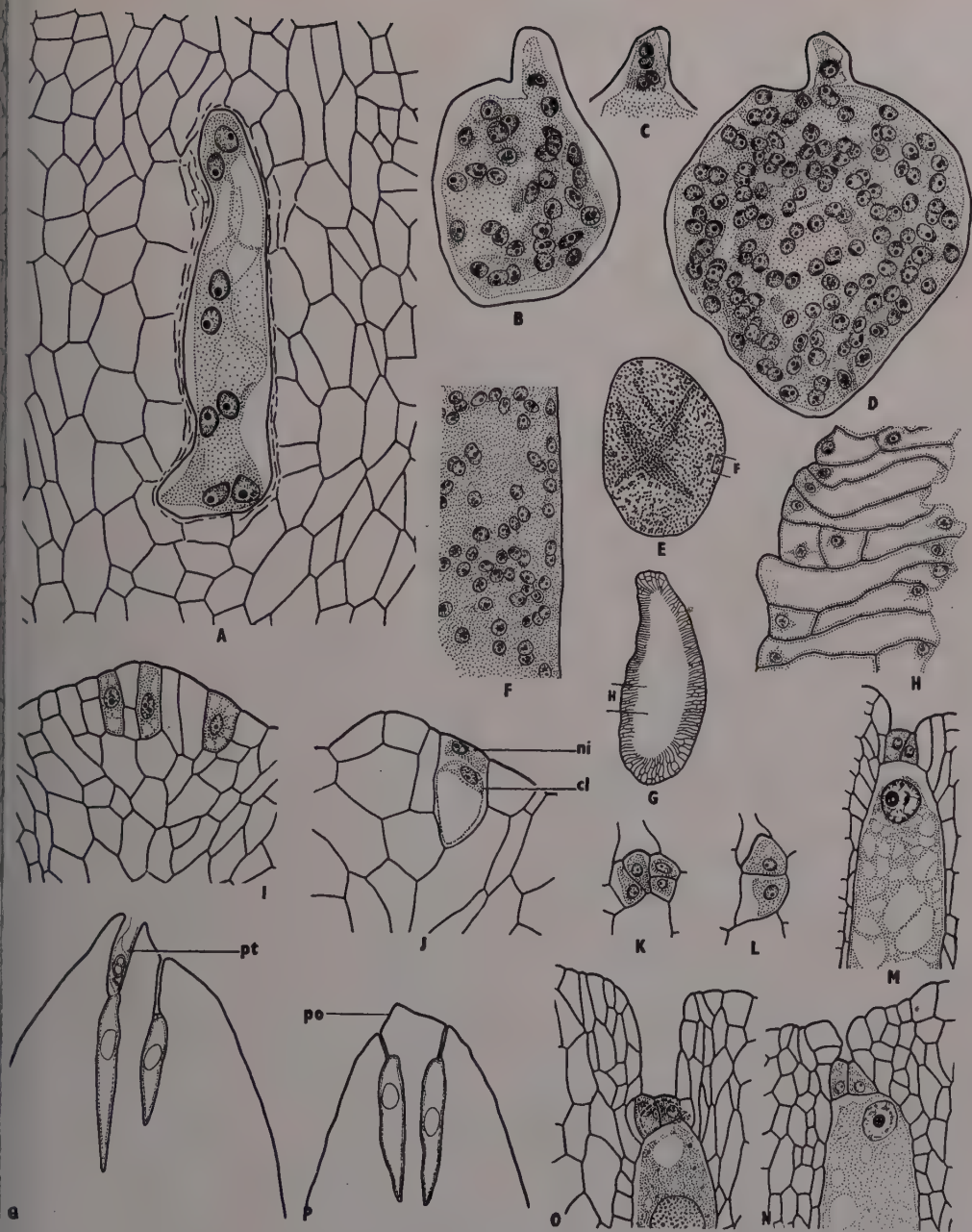
Fig. 12A-Q — Female gametophyte and archegonium. (*cl*, central cell; *ni*, neck initial; *po*, protuberance of the gametophyte; *pt*, pollen tube). B-F are from whole mounts. A. L.s. portion of nucellus containing an 8-nucleate gametophyte. × 320. B. 32-nucleate gametophyte. × 139. C. Micropylar projection of female gametophyte showing four free nuclei. × 139. D. 128-nucleate gametophyte. × 139. E. Free nuclear gametophyte; late stage. × 12. F. Portion marked F in E, enlarged. × 139. G. L.s. gametophyte in which wall formation has been initiated. × 12. H. Portion marked H in G enlarged. × 139. I. Archegonial initials. × 139. J. Division of an archegonial initial to form the neck initial and the central cell. × 139. K, L. Abnormal disposition of neck cells. × 139. M-O. Longisections of the micropylar region of the female gametophyte to show the formation of the archegonial chamber. × 139. P, Q. L.s. micropylar region of female gametophytes. Note the protuberance (*po*) at the upper end. A pollen tube has entered the archegonium on the left in Q. × 33.
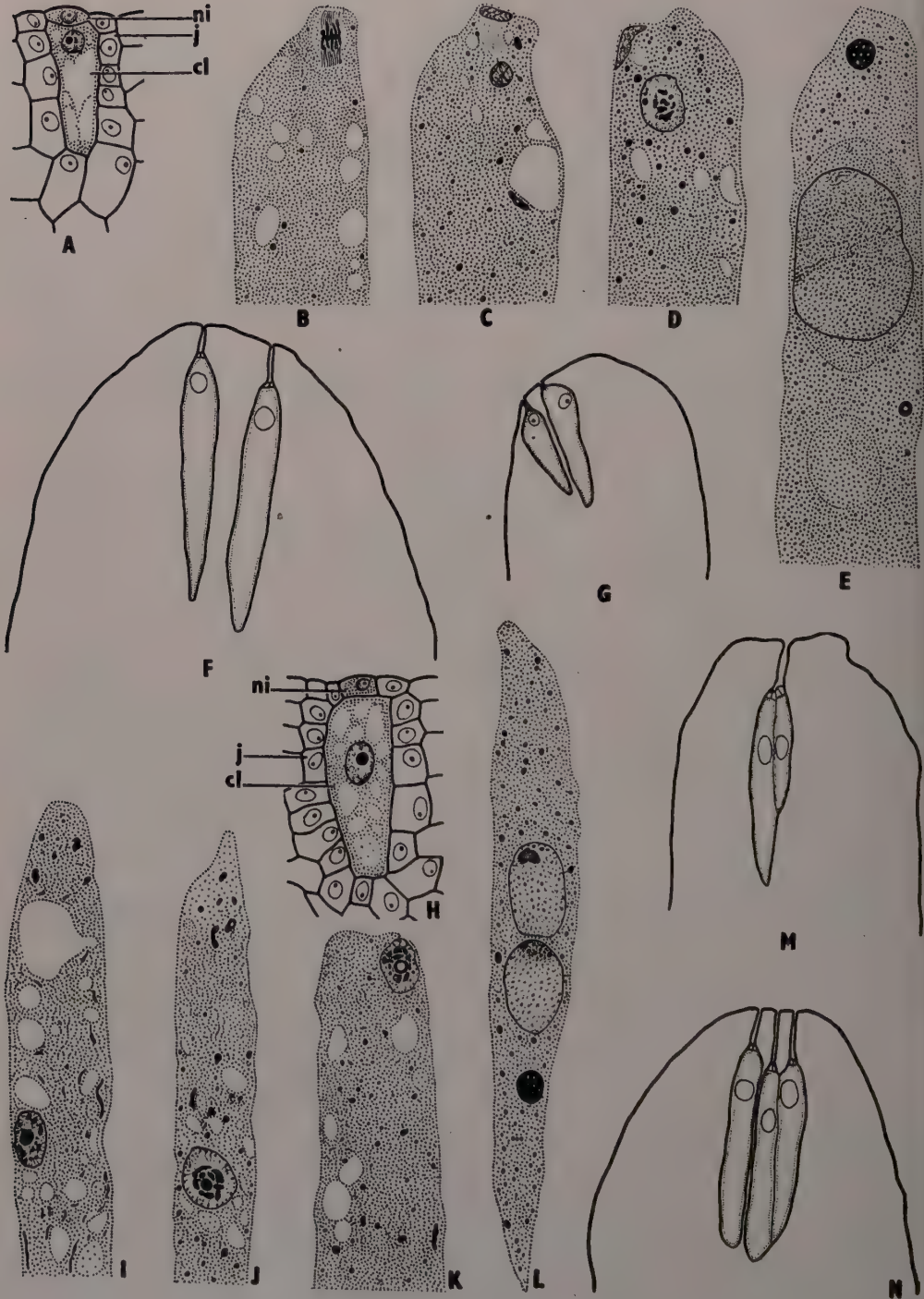
the pollen tube is seen near the female gametophyte when the archegonia are still in the foam stage. In such gametophytes the archegonium with a pollen tube above it (since each archegonium has its own cavity above it) matures earlier than the other archegonia of the same gametophyte. This points to some correlation between the nearness of the pollen tube and the maturation of the archegonium.

The presence of archegonia in a complex is characteristic of the families Cupressaceae and Taxodiaceae. Rarely in *Cephalotaxus*, two or three archegonia are closely adjacent (Fig. 13N) and sometimes have a common archegonial cavity (Fig. 13M), thus somewhat approaching the condition found in the above mentioned families.

Fertilization

The pollen tube discharges the male gametes inside the archegonium. As pointed out earlier, the tube and the stalk nuclei degenerate and are never seen in the cytoplasm of the egg. One (Fig. 15E) or both (Fig. 14A, B) the gametes may enter an archegonium, always carrying a portion of the male cytoplasm (Fig. 14B). As the male nucleus approaches the female, the cytoplasm in the upper part of the archegonium becomes vacuolated (Fig. 14B-D). When the two gametes come in contact with each other, the male nucleus becomes lenticular and both become enveloped by the male cytoplasm (Fig. 14D, E). The nuclear membranes dissolve at the point of contact and the chromatin of the two nuclei approaches each other (Fig. 14E). Numerous fibrils appear in the fusion nucleus. The two

chromatin groups merge (Fig. 14F) and the fibrils become arranged in a bipolar spindle (Fig. 14G). Soon the chromosomes are seen in a metaphase plate which is slightly oblique to the long axis of the archegonium (Fig. 14G).

Some gametophytes showed two proembryos and only one pollen tube. The proembryos were produced from adjacent archegonia which were probably fertilized by the same pollen tube. Two cases of polyspermy have been observed. Here a supernumerary pollen tube had discharged the male gametes into a two-nucleate proembryo (Fig. 14H).

Embryogeny

The first division of the zygote nucleus is intranuclear and takes place *in situ*. The resulting two nuclei are enclosed in the male cytoplasm and migrate to the lower part of the proembryo (Fig. 15A, B). Rarely the nuclei separate and only one of them moves down (Fig. 15C). The path, which the nuclei take during their migration to the base of the proembryo, is marked by numerous vacuoles in the cytoplasm (Fig. 15C-E). After reaching the lower portion of the proembryo the two nuclei divide simultaneously and the resulting four nuclei separate from each other due to the disappearance of the sheath of male cytoplasm (Fig. 15D, E). By this time the upper portion of the proembryo becomes much vacuolated, while the lower portion has dense cytoplasm with numerous protein globules. The two regions are separated by a large vacuole. The four nuclei divide simultaneously (Fig. 15F) and the resulting eight nuclei

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FIG. 13A-N — Female gametophyte and archegonium. (*cl*, central cell; *j*, jacket layer; *ni*, neck initial). A. A young archegonium. × 182. B. Division of the nucleus of the central cell. × 182. C. Newly formed ventral canal and egg nuclei. × 182. D. Enlargement of the egg nucleus and the degeneration of the ventral canal nucleus. Small granules are present in the peripheral part of the egg nucleus. × 182. E. Upper portion of the mature egg cell. × 182. F. L.s. gametophyte showing mature archegonia. × 42. G. Gametophyte bearing somewhat lateral archegonia. × 42. H, I. Young and old archegonia with the nucleus of the central cell in their middle. × 182. J, K. Nucleus of the central cell enlarging to form the egg nucleus, without cutting off the ventral canal nucleus. × 182. L. Two-nucleate egg. × 182. M, N. L.s. gametophytes showing archegonia in a complex. × 42.
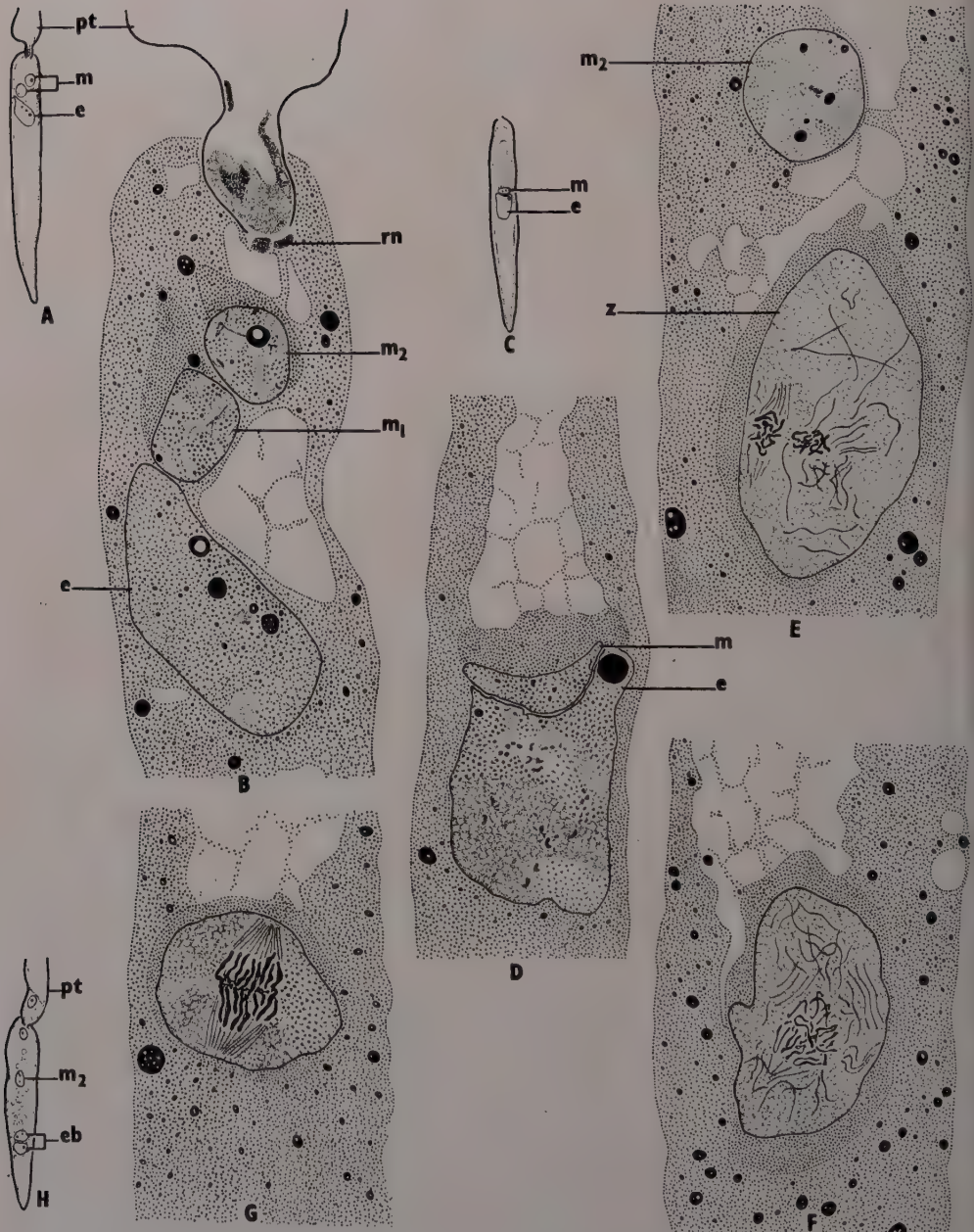
Fig. 14A-H—Fertilization. (e, egg nucleus; eb, proembryonal nuclei; m, male nucleus; m_1, first male nucleus; m_2, second male nucleus; pt, pollen tube; rn, remnants of the neck cells; z, zygote nucleus). A. L.s. archegonium into which the pollen tube has discharged two male gametes. × 37. B. Enlarged view of the upper portion of A. × 382. C. Outline diagram of D. × 37. D. Middle portion of the archegonium showing lenticular male nucleus. × 382. E-G. Stages in the mixing of the male and female chromatin. The second male gamete is seen in E. × 382. H. L.s. proembryo into which a second pollen tube has discharged the male gametes. × 37.

are arranged irregularly (Fig. 15G). One more simultaneous division occurs (Fig. 15H) and wall formation is initiated at the 16-nucleate stage. The size of the nuclei becomes progressively smaller as the proembryo advances in age (cf. Fig. 15B-G).

A varying number of large nuclei are also observed in the upper portion of proembryo. Eventually all these nuclei degenerate but during this process some fibres appear around them (Fig. 15K). They may arise from one or more of the following sources —

(i) *The second male gamete and its derivatives*: When both the male nuclei enter the archegonium, one fuses with the egg nucleus, while the second may give rise to supernumerary nuclei (Figs. 14E, 15C). The division of the male gamete may be amitotic (Fig. 15I) or mitotic and synchronous with that of the proembryonal nuclei in the lower portion of the archegonium (Fig. 15D).

(ii) *Extra sperms*: These may be derived from an additional pollen tube.

(iii) *Persisting ventral canal nucleus*: This happens only rarely (Fig. 15C, E).

(iv) *Relict and lagging nuclei*: After the four-nucleate stage of the proembryo, its nuclei are sometimes extruded toward the upper part (Fig. 15J). Rarely only one of the two proembryonal nuclei migrates to the base. The nucleus that remains behind may give rise to the supernumerary nuclei.

Rarely a proembryo shows three nuclei (Fig. 15L). This may be due to the division of only one of the nuclei of the two-nucleate proembryo or the extrusion of one nucleus from a four-nucleate proembryo. A six-nucleate proembryo (Fig. 15M) may originate either from one which is three-nucleate or by the extrusion of two nuclei, or by failure of division of two of the nuclei of the four-nucleate proembryo.

Wall formation in the proembryo results in the formation of a lower embryonal group of 10-13 small cells and an upper tier of three to six cells open toward the archegonium (Fig. 16A). The lowermost one or two cells of the embryonal group are large, have dense cytoplasm and a prominent nucleus and constitute the ' cap cells '. There is another division in

the embryonal group increasing the number of cells to 20-26. The upper tier divides transversely (Fig. 16B) to form the lower prosuspensor tier and an open tier which degenerates after some time (Fig. 16C-F). By this time the cytoplasm of the archegonium also degenerates to form a dense mass or ' plug ' (Fig. 16C-F, J). The prosuspensor tier elongates (Fig. 16D-G) and pushes the embryonal tier into a cavity in the centre of the female gametophyte (Fig. 16H). This cavity is formed by the degeneration of cells and progresses toward the chalazal end. Some of the prosuspensor cells divide transversely and the cells cut off by them toward the top (Fig. 16F) may give rise to numerous small embryos[4] (Fig. 16F, G, K). Sometimes these embryos grow quite large and develop a secondary suspensor (Fig. 17B) but generally degenerate during the development of the seed. All the cells of the young emhryo contain starch.

By the time the prosuspensor has fully elongated, the cap cells degenerate and their place is occupied by three or four adjacent embryonal cells which enlarge somewhat (Fig. 16G). These cells also degenerate after some time. Rarely a cap cell may be binucleate (Fig. 16 I). The embryo presented in Fig. 16J is abnormal in that the prosuspensor-embryos are absent and the prosuspensor has twisted through an angle of 180°.

The basal cells of the embryonal mass elongate to form the secondary suspensor (Fig. 16K), which pushes the embryonal mass further down into the female gametophyte. The prosuspensor cells collapse by this time. Occasionally the cells of the secondary suspensor proliferate to form a small embryo. The cleavage of the embryonal mass is very rare and has been observed only in three cases.

As the embryonal mass grows a massive suspensor is formed. Out of the one or two embryonal masses which are generally derived from as many archegonia one ultimately takes the lead and develops into a mature embryo.

The leading embryo is undifferentiated and grows by predominant periclinal

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4. For convenience these embryos have been later referred as prosuspensor-embyros.
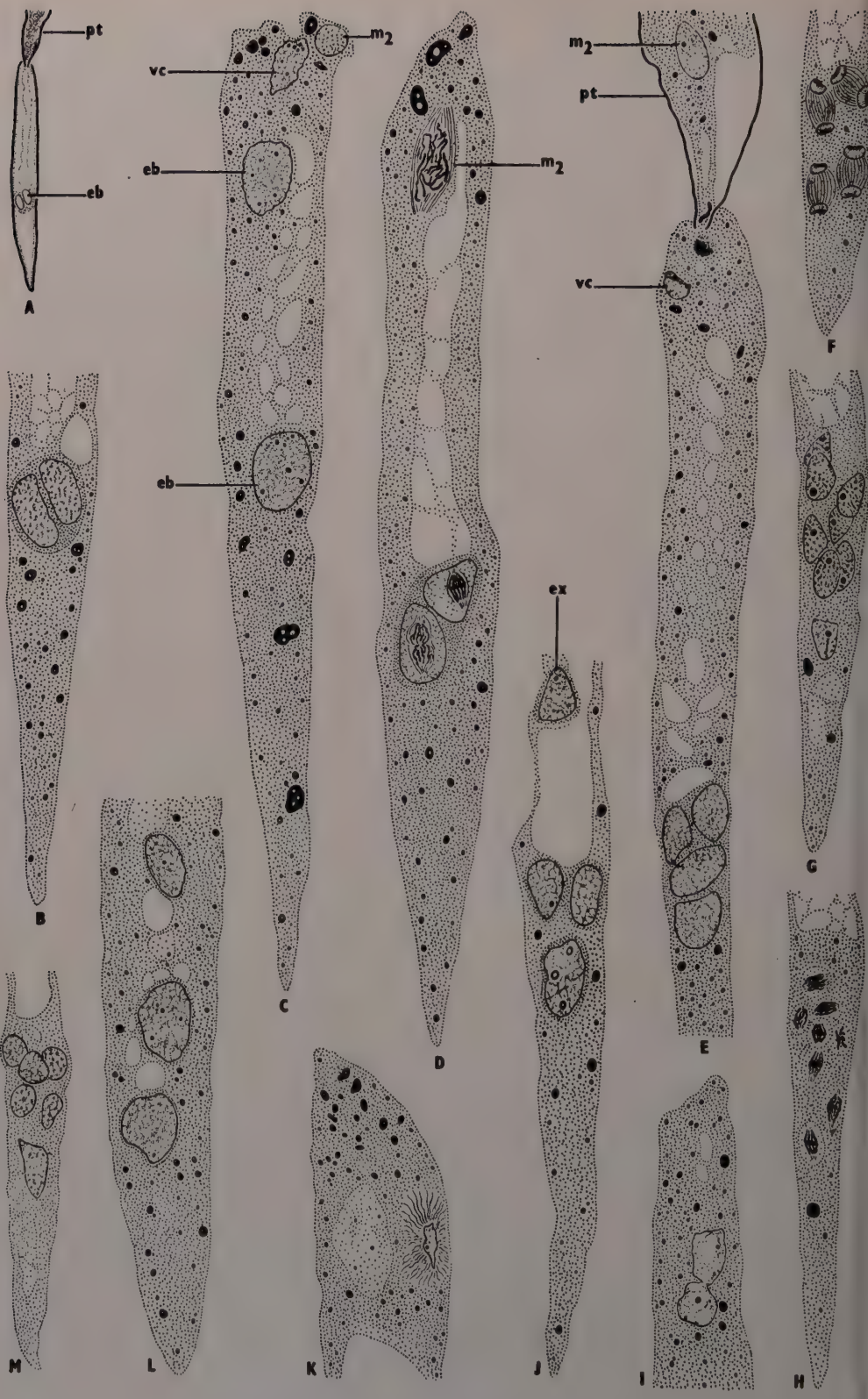
divisions in its outermost cell layer (Fig. 17C). Soon the usual three layers — dermatogen, periblem and plerome — and the root initials become distinct (Fig. 17D). The two cotyledonary primordia appear at this time and grow quickly (Fig. 18A). In the mature embryo the root tip initials are very distinct (Fig. 18B) while the stem tip is flat and undifferentiated (Fig. 18C). The various histological regions of the stem tip become recognizable only when the seed germinates (Fig. 18D).

According to Buchholz (1925) the central region of the hypocotyl of the mature embryo degenerates in *Cephalotaxus fortunei*. This must be considered to be only exceptional since such a behaviour is not shown by the embryo of any other conifer. Like all other conifers, in *C. drupacea* the central region of the hypocotyl remains healthy.

Seed and Seed Coat

At the time of pollination the integument consists of several layers of thin-walled cells. Some of these cells contain tannin. As the mother cell is differentiating in the ovule, resin ducts appear in the chalazal part of the integument (Fig. 11B). These ducts are narrow in diameter and are lined by an inner layer of epithelial cells and an outer layer of tannin cells. The lower part of the integument becomes meristematic and consists of many layers of isodiametric cells which contribute a major share to the mature seed coat. At the free nuclear stage of the gametophyte the lower portion of the integument enlarges considerably. The innermost cells of the integument are narrow and vertically elongated; these are crushed by the enlarging gametophyte (Fig. 19A). Outer to this zone are patches of tannin cells and then a number of layers of parenchyma. A band of tannin cells is very prominent in the central region of the integument. Stomata are present in the epidermis (Fig. 19A, C). During the maturation of the archegonium and early embryogeny, some of the hypodermal cells of the integument acquire thick, pitted walls and a layer of cells inner to the tanniniferous band elongates radially (Fig. 19B, C), undergo periclinal divisions, and together with 7-8 layers of cells form the sclerenchymatous stony layer which is difficult to section in later stages (Fig. 19D, E). Cells outer to the stony layer form the red fleshy layer which contains scattered thick-walled and tannin cells and is traversed by two vascular bundles and numerous resin ducts. The epithelial lining of the latter degenerates by this time (Fig. 19E). The cells inner to the stony layer form the papery layer which has a mosaic surface due to the presence of patches of tannin cells (Fig. 19F, G).

When the seed matures on the plant, it is large and consists of a thick coat, and an embryo extending to only a small length of the massive female gametophyte (Fig. 19H). On shedding the outer fleshy layer of the seed coat soon disintegrates and the seed is now covered by the stony layer which is sharply pointed at the two ends. Meanwhile the embryo grows further and finally extends almost the entire length of the gametophyte

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Fig 15A-M — Embryogeny. (*eb*, embryonal nuclei; *ex*, extruded nucleus; m_2, second male gamete; *pt*, pollen tube; *vc*, ventral canal nucleus). A. L.s. two-nucleate proembryo. × 42. B. Lower portion of A enlarged. × 194. C. The two proembryonal nuclei have separated from each other and one of them is migrating down. Note the persisting ventral canal nucleus. × 194. D. The two proembryonal nuclei dividing. The second male gamete is also dividing in the upper portion of proembryo. × 194. E, G. Four- and eight-nucleate proembryos. The second male gamete has remained behind in the pollen tube in E. × 194. F, H. The nuclei of the four- and eight-nucleate proembryos dividing. × 194. I. The upper portion of the proembryo showing amitotic division. × 194. J. Extrusion of one of the proembryonal nuclei. × 194. K. The upper portion of the proembryo showing degeneration of a nucleus. × 194. L, M. Three- and six-nucleate proembryos. × 194.
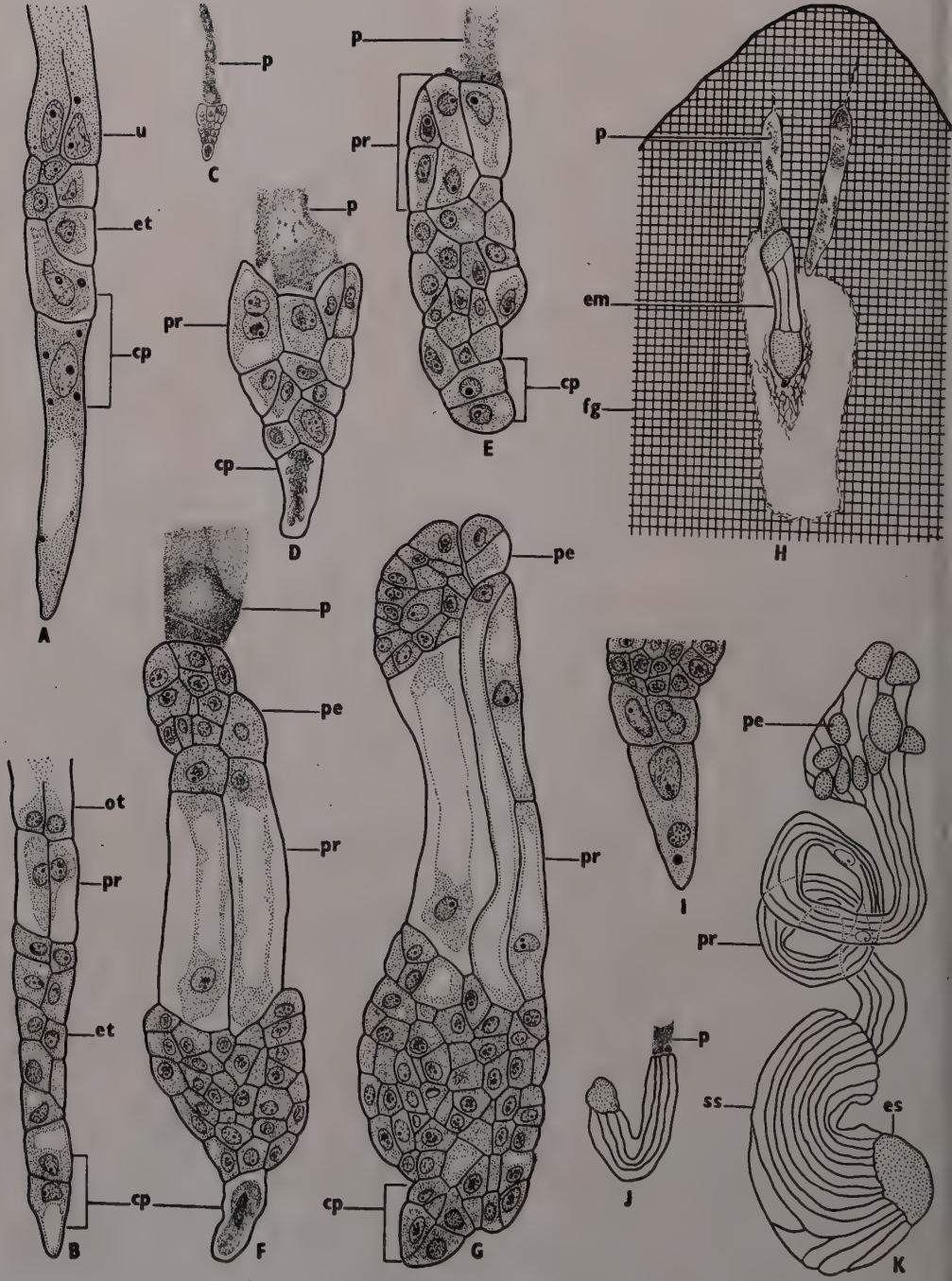
(Fig. 19 I, J).   The seeds undergo a long period of dormancy lasting for two to three years.

Seedling

The stone of the seed is somewhat compressed and pointed at both ends, the chalazal end being sharper (Fig. 20A). During germination the stone cracks longitudinally on the two sides and the radicle emerges out (Fig. 20B, C).   The tap root branches very soon (Fig. 20D-G). The stony layer is generally cast off from the seedling along with the female gametophyte at a later stage.   In common with other conifers, germination is of the epigeal type (Fig. 20E-H).   The hypocotyl and the cotyledons are fleshy (Fig. 20H). The juvenile leaves are spirally arranged and are longer but narrower than the mature leaves.   The cotyledonary leaves persist for more than two years after germination (Fig. 20H).

ANATOMY — The primary root shows a diarch structure with two distinct patches of exarch xylem alternating with two elongated strips of phloem.   The entire length of the hypocotyl shows the typical root structure (Fig. 21A).   A group of large parenchymatous cells is present outside each patch of phloem and the cortical cells are full of starch.   The vascular transition begins just below the cotyledonary node.   The two xylem patches unite to form a mass (Fig. 21B). At a slightly higher level the xylem tracheids in the centre are replaced by parenchymatous cells to form the pith which gradually becomes wider (Fig. 21C, D).   Meanwhile centrifugal xylem elements start appearing and the xylem ring becomes mesarch (Fig. 21C, D).   Due to further replacement of centripetal xylem by pith, the xylem becomes fully endarch (Fig. 21E, F).   The phloem also now forms a complete ring (Fig. 21E) and a group of large parenchymatous cells extends outside the phloem adjacent to the two patches of protoxylem (Fig. 21F). At this level the vascular ring breaks up into two large groups alternating with two small ones (Fig. 21F).   The latter enter the hypocotyl after branching.   The two large bundles lie in direct line above the protoxylem poles of the root and they supply the cotyledons.   Thus the vascular transition in the seedling occurs between the root and the cotyledons.

The cotyledons look plano-convex in cross-section and the mesophyll cells are full of starch grains (Fig. 22A, B).   The stomata are confined to the adaxial surface.   At the base of the cotyledons, the bundle is kidney-shaped and has a strictly endarch xylem but higher up it becomes tangentially elongated and centripetal xylem tracheids appear (Fig. 22 B). These centripetal tracheids are short and have only spiral thickenings (Fig. 22C).   A group of large parenchymatous cells lies next to the phloem.

The leaves following the cotyledons show a narrow outline and have an undifferentiated mesophyll (Fig. 22D). Stomata are restricted to the abaxial surface.   The vascular bundle is not associated with transfusion tracheids (Fig. 22E) but contains some centripetal xylem elements (Fig. 22E, F).

FIG. 16A-K — Embryogeny. (*cp*, ' cap cells '; *em*, embryo; *es*, embryonal mass; *et*, embryonal group; *fg*, female gametophyte; *ot*, open tier; *p*, plug; *pe*, prosuspensor-embryo; *pr*, prosuspensor tier; *ss*, secondary suspensor; *u*, upper tier).   I-K have been drawn from whole mounts.   A. L.s. proembryo after wall formation.   The nuclei of the upper tier are in prophase. × 163.   B. Proembryo showing the open tier, prosuspensor tier, and the embryonal group. × 163.   C. Outline diagram for D; the upper narrower portion represents the degenerated remains of the egg cytoplasm. × 37.   D. Lower portion of C enlarged.   The open tier has degenerated. × 163. E. The cells of the prosuspensor tier have divided transversely. × 163.   F. The cap cells have degenerated and prosuspensor-embryos have been initiated. × 163.   G. Cap cells (*cp*) derived from the embryonal mass. × 163.   H. L.s. micropylar region of female gametophyte showing the embryo in the cavity formed by the disintegration of the cells of the gametophyte. × 37.   I. Lower portion of the embryo showing two-nucleate cap cells. × 163.   J. Abnormal embryo. × 37.   K. Origin of the secondary suspensor (*ss*); note embryos (*pe*) formed from prosuspensor cells. × 37.
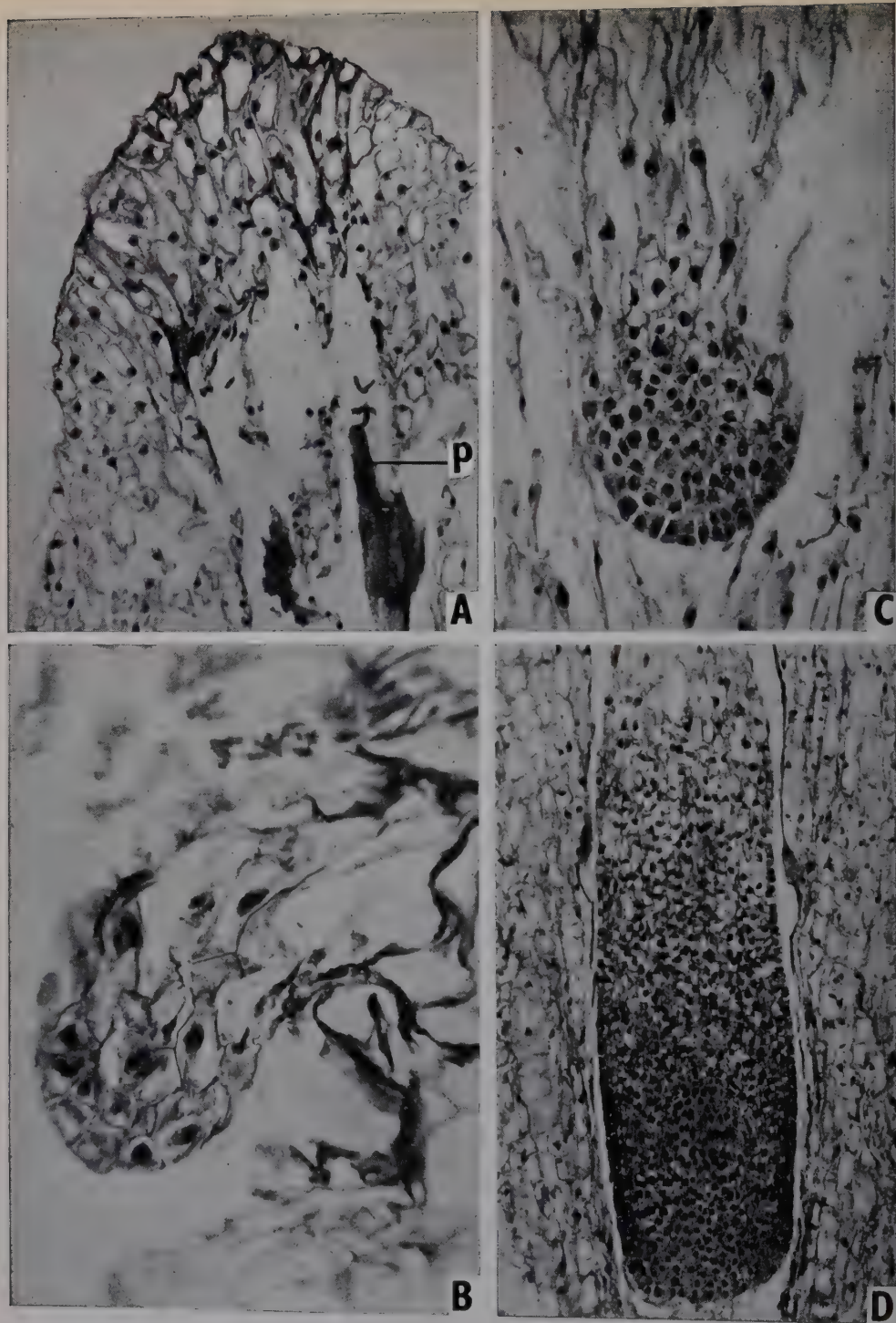
FIG. 17A-D — Embryogeny. (p, plug). A. L.s. micropylar region of the female gameto-phyte showing multinucleate and richly cytoplasmic cells. × 94. B. Prosuspensor-embryo showing suspensor. × 235. C. Young embryo; note periclinal divisions in the outermost layer. × 95. D. Embryo showing differentiation of root tip and cotyledonary primordia. × 60.

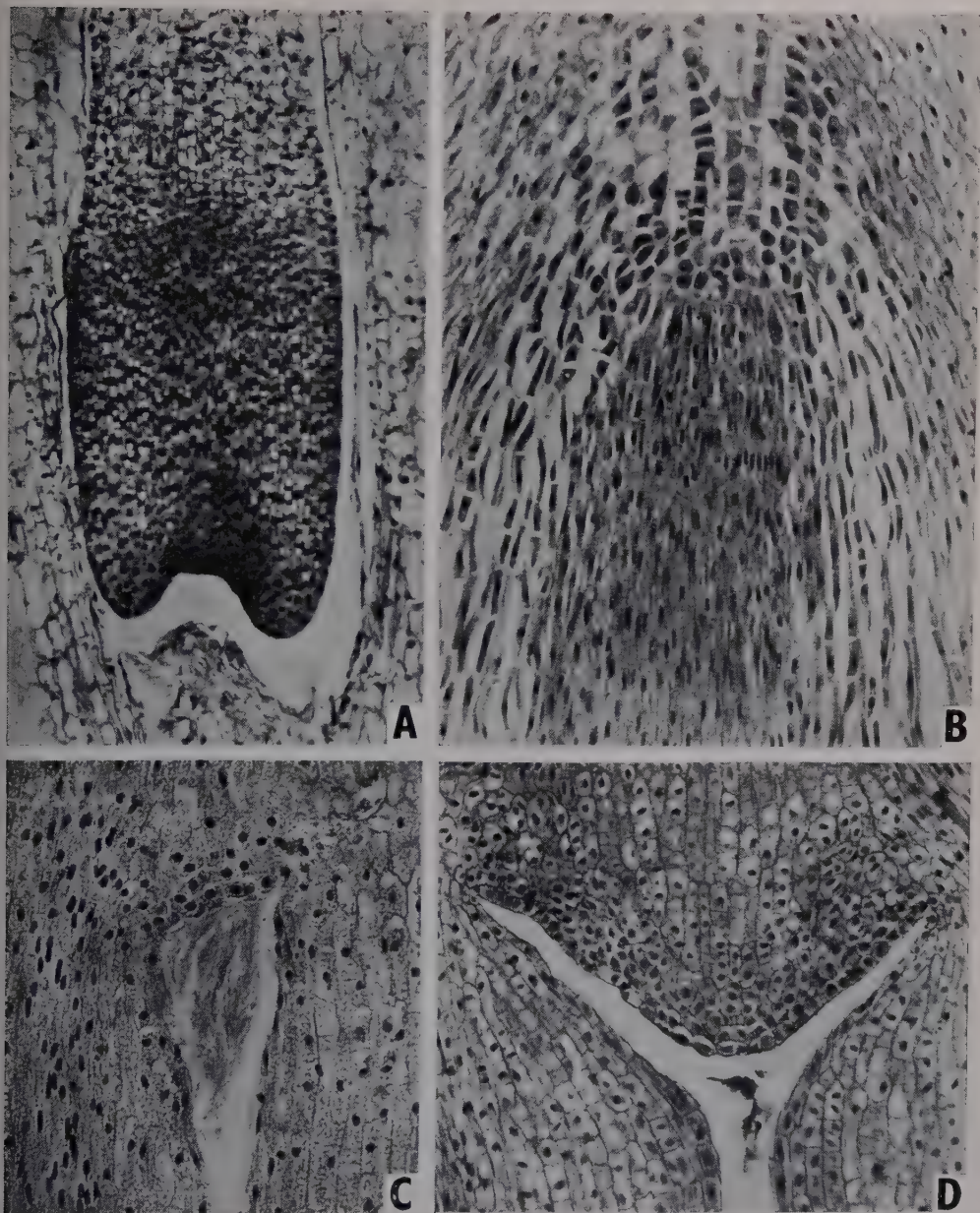Fig. 18A-D — Embryogeny. L.s. embryo showing cotyledons. × 60. B. L.s. root tip at the time of seed shedding. × 78. C. L.s. middle part showing relatively undifferentiated stem tip, and cotyledons. × 78. D. Shoot tip at the time of seed germination. The usual four cytohistological zones have been formed. × 78.
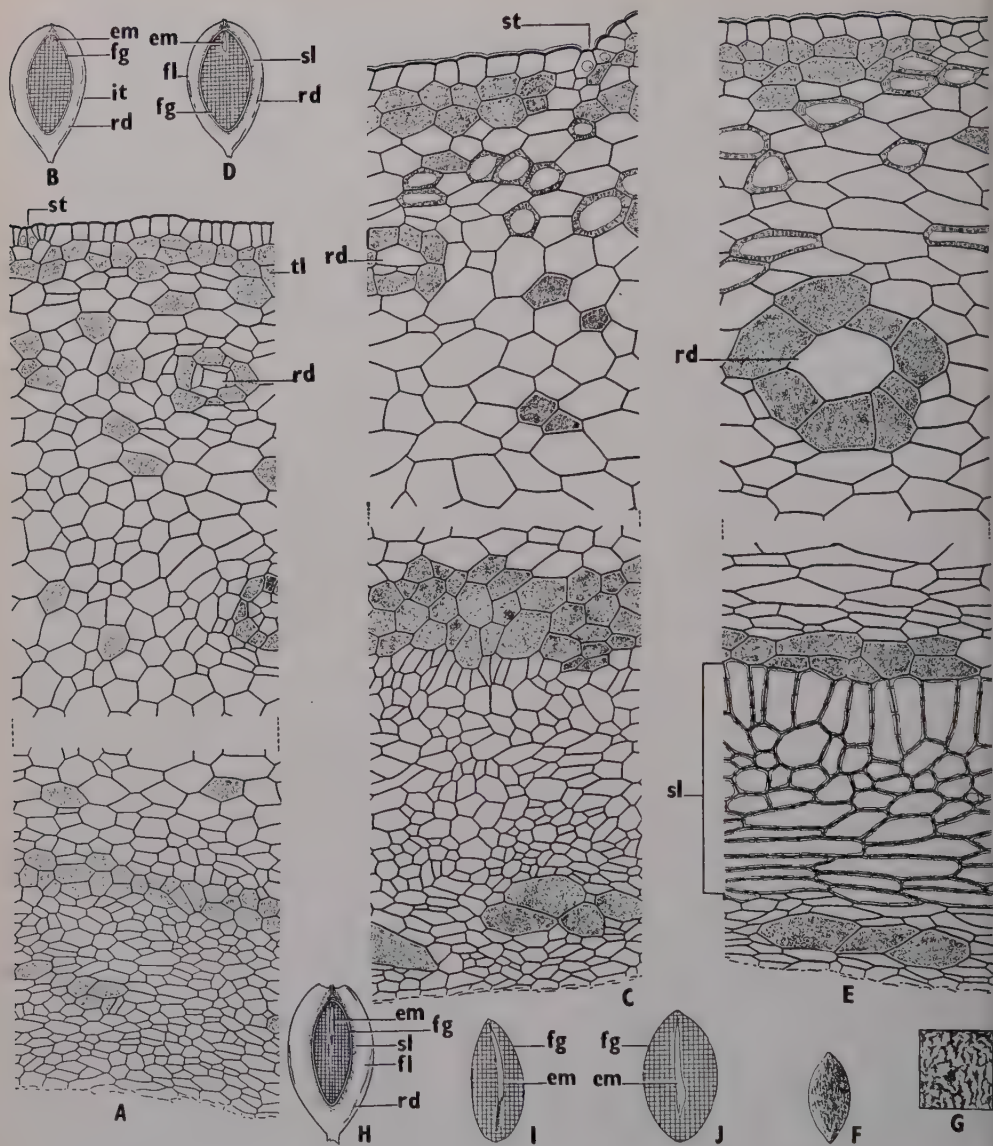
Fig. 19A-J — Seed and seed coat (*em*, embryo; *fg*, female gametophyte; *fl*, fleshy layer of the integument; *it*, integument; *rd*, resin duct; *sl*, stony layer of the integument; *st*, stoma; *tl*, tannin cell). A. T.s. portion of the integument at the free nuclear gametophyte stage. × 105. B, D. Longisections of seeds containing embryos in different stages of developmene × 1. C, E. T.s. integument at the stages of the ovule shown in B and D respectively. × 105. F. Seed from which the fleshy and the sotny layers of the integument have been removed. Note the mosaic surface of the papery layer. × 1. G. Surface of the same enlarged. × 4. H. L.s. seed at the time of shedding. × 1. I, J. Longisections female gametophyte from seeds shed from the plant. Note the elongation of embryo × 1.
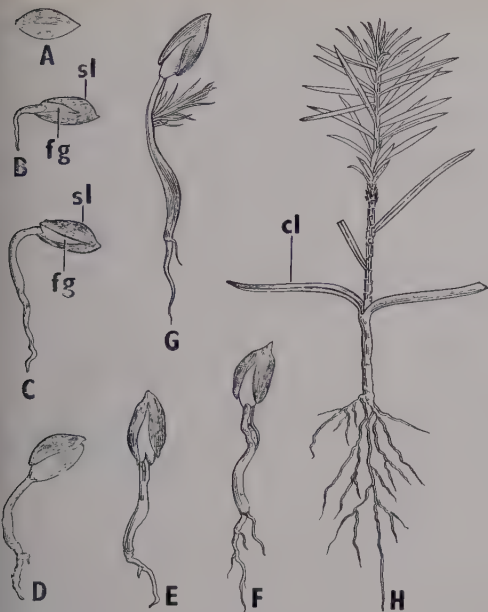
FIG. 20A-H — Seed germination. (*cl*, cotyledonary leaf; *fg*, female gametophyte; *sl*, stony layer). A. The seed with its stony layer. × 0·5. B-G. Progressive stages in the germination of the seed. × 0·5. H. Two years old seedling. × 0·5.

Chronology of Reproductive Phases

Figure 23 summarizes the sequence of occurrence of the various developmental stages at different seasons of the year.

MALE CYCLE — The short lateral shoots which later bear the male cones arise in the middle of June. The male cones are initiated in the last week of August and the microsporangia appear in the beginning of September. The sterile region of the microsporophyll and the wall layers of the microsporangia are fully developed by the end of this month. Between October and the first half of December the microsporangia enlarge and the sporogenous cells multiply and differentiate into the microspore mother cells. The tapetum becomes distinct by the middle of October and many of its cells are binucleate by the middle of December. The microsporangia undergo a rest period of about two and a half months, starting from the middle of December to the first week of March. The microspores separate from the tetrads

and undergo the first division during the second week of March. The sporangia start dehiscing by the third week of March and the shedding period extends till the beginning of April.

The pollen grain produces a short pollen tube within a month of its landing on the ovule. The antheridial cell divides by the third week of April and the stalk and the body cells soon migrate into the lower portion of the pollen tube which now undergoes a period of rest for about a year. In the beginning of the next April it grows through the nucellus and reaches the female gametophyte by the second week of the month. The division of the body cell was found to take place between the 10th and 18th of April and the male gametes are liberated into the archegonium soon after.

FEMALE CYCLE — The female cones are initiated in the middle of October and the ovular primordia and integument appear about a month later. By the middle of December the sporogenous cells become situated below three or four layers of nucellar cells, and a few cells of the integument show tannin. The female cone overwinters in this condition. Favre-Duchartre (1957) failed to observe the tannin cells in the integument at this stage and, therefore, described it as homogeneous.

All these developments occur while the female cone is still covered by the bud scales. During the first week of March the cones emerge out of the scales and the pollination drop is seen on the micropyles of the ovules from March 12 to March 20. By this time the sporogenous tissue becomes deeply embedded in the nucellus. About a month after pollination, i.e. the middle of April, the megaspore mother cell becomes distinct from the sporogenous cells (Fig. 11B, C) and the micropyle becomes closed due to the ingrowth of inner epidermal cells of the integument.

The mother cell undergoes meiosis by the middle of November and the nucleus of the functional megaspore divides three or four times producing 8 or 16 nuclei. The pavement tissue is very well developed and packed with starch grains at this time. In this stage the ovule rests for a second winter and enlarges in the coming spring
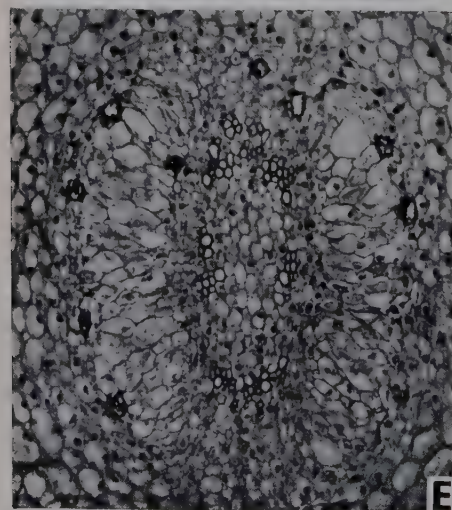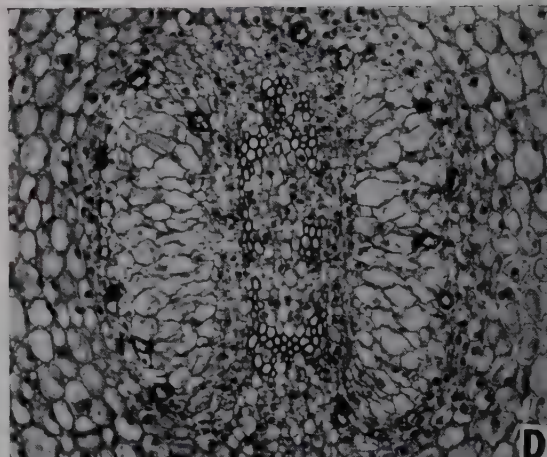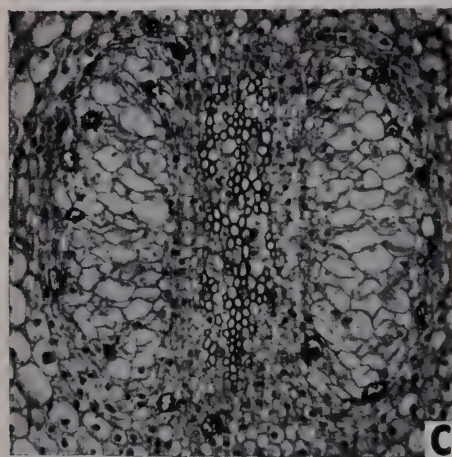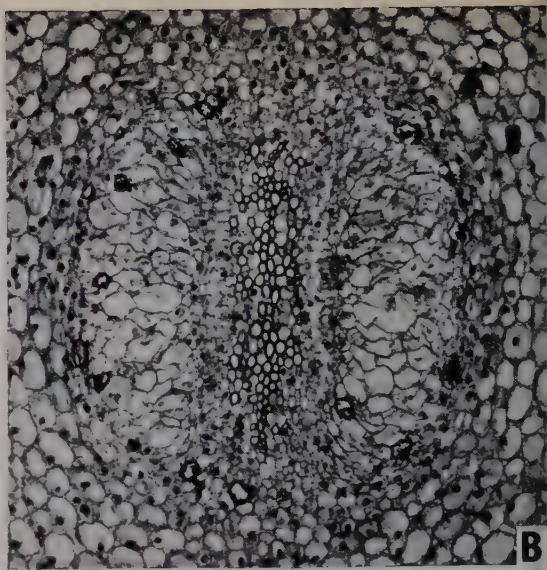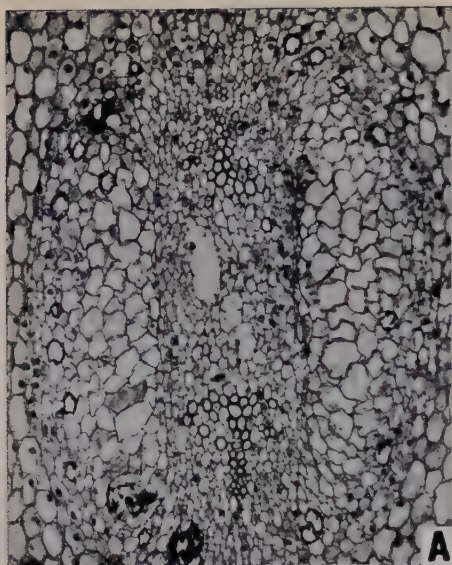
when a cellular gametophyte is organized. The archegonial initials make their appearance in the first week of April. In the second week the nucleus of the central cell divides and the archegonia mature in the next two or three days. Coker (1907) has, however, described that this process extends over 10 days. Stages of fertilization are seen between April 12 to April 20 and the embryo starts developing soon after. By the end of May the cotyledons differentiate, large quantities of food accumulate in the female gametophyte and the middle portion of the integument becomes stony. The seeds mature by the third week of July.

The ovule requires about one year and nine months from its appearance to its maturation into a seed.

Discussion

One vascular trace is received by every lateral appendage like leaf, bract, bud scale and microsporophyll. Three traces, one anterior and two lateral, are present at the base of the female cone, the bract and its ' seed-scale complex ', the male cone and the lateral shoot which bears the male cones. The anterior bundle as such supplies the axilant leaf, while the two lateral bundles divide to form a ring which supplies the axillary organs. There is no doubt that the male and female cones, and the short lateral shoot bearing the male cones are all morphologically equivalent to shoots. Each of these receives three vascular traces from the central stele. It may well be generalized that shoot-like organs of *Cephalotaxus* receive three vascular bundles. The ' seed-scale complex ' represents a shoot, since it also receives three vascular bundles. The ontogenetical evidences are quite in support of this hypothesis. The segmentation of the outgrowth in the axil of each bract of the female cone is very much like that of the apex of any vegetative shoot, the only difference being that the growth of this

structure is extremely limited. After studying several abnormal cones Worsdell (1901) concluded that this outgrowth represents a secondary axis. Similar conclusions have also been reached by Hirmer (1936). Hence the female cone of *Cephalotaxus* is biaxial.

It is a debatable point whether the male gametes are cells (Coker, 1907; Sugihara, 1947; Favre-Duchartre, 1957) or nuclei (Arnoldi, 1900; Lawson, 1907; Kaur, 1958). None of the earlier investigators had observed the division of the body cell. According to present observations the nuclear division of the body cell is not followed by wall formation, so that only two male nuclei are formed.

According to the previous workers the ovule is homogeneous till a megaspore mother cell differentiates. Present work shows that the archesporial cells are hypodermal and can be recognized in the ovule at an early stage.

Kaur (1958) writes for *C. drupacea* var. *pedunculata*: " A single megaspore mother cell is organized. A linear ' tetrad ' is formed but the upper diad mostly remains undivided." However, in my material the upper dyad cell was always seen to divide, though later than the lower. Thus a normal tetrad is consistently present.

According to Favre-Duchartre (1957) the characteristics of the egg nucleus are the same as those of the central cell nucleus. I find the central cell nucleus to be densely chromatic with the chromatin in the form of threads and granules, and having a prominent nucleolus. The egg nucleus, on the other hand, mostly contains nucleoplasm in which the nucleolus is absent and the chromatin reduced to a dot-like body.

According to Arnoldi (1900) the upper part of the cytoplasm of the egg swells and exerts pressure on the neck cells, thus forcing open the archegonium and bringing it nearer to the male gametes. However, the present observations reveal that the archegonium opens due to a degeneration

FIG. 21, A-F — Seedling anatomy. A. T.s. central portion of hypocotyl. × 70. B-F. Transections of cotyledonary node from hypocotyl upward, showing vascular transition. × 70.

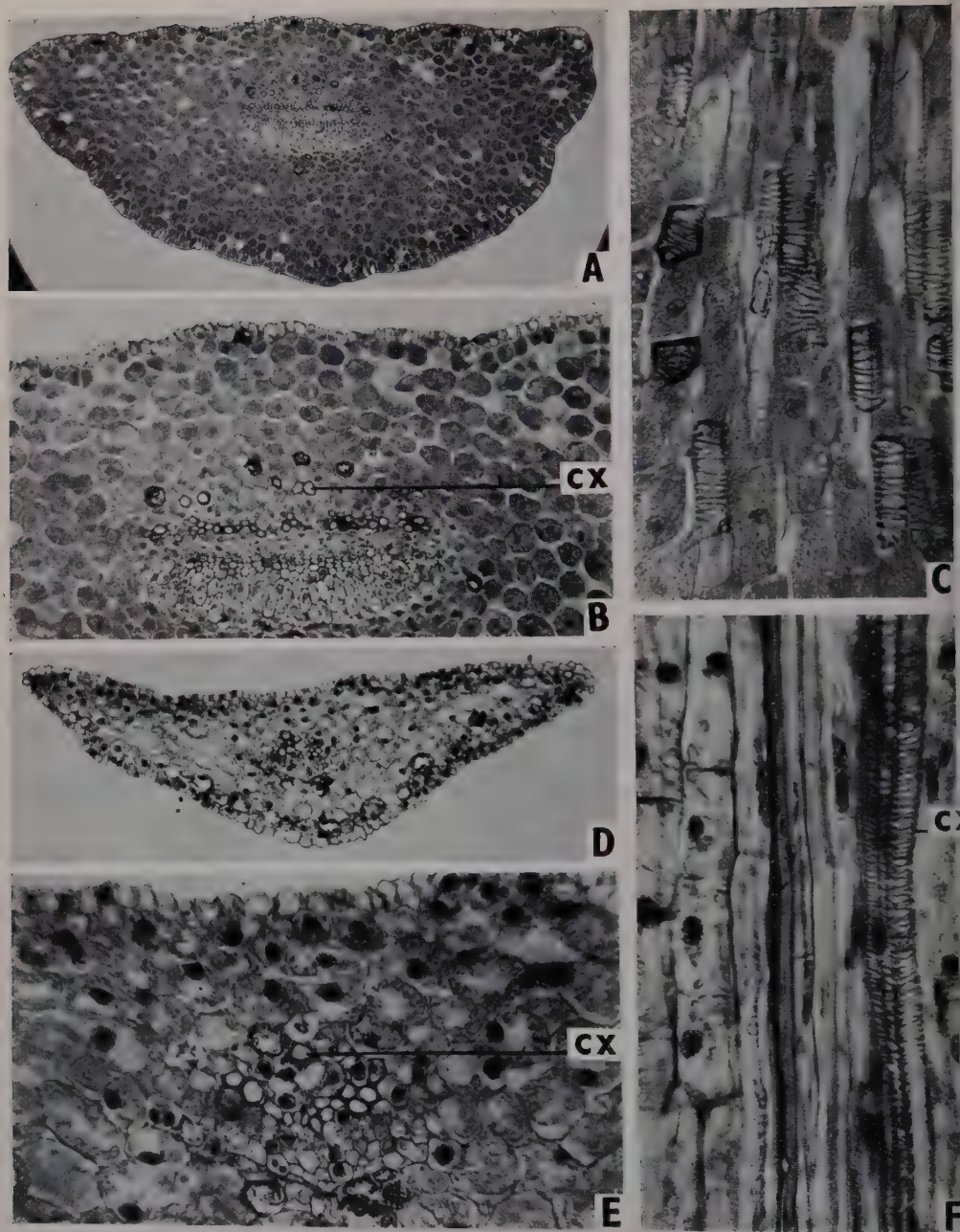Fig. 22, A-F — Seedling anatomy. (*cx*, centripetal xylem). A. T.s. cotyledonary leaf. × 32.
B. Vascular bundle of the same enlarged. Note a few centripetal xylem elements and stomata
on the adaxial surface. × 64. C. Centripetal xylem from a section passing parallel to the sur-
face of a cotyledonary leaf. × 198. D. T.s. juvenile leaf. × 59. E. Vascular bundle of the
same enlarged. Note a centripetal xylem element in addition to the normal centrifugal xylem.
× 201. F. L.s. part of the bundle showing centripetal xylem. × 205.
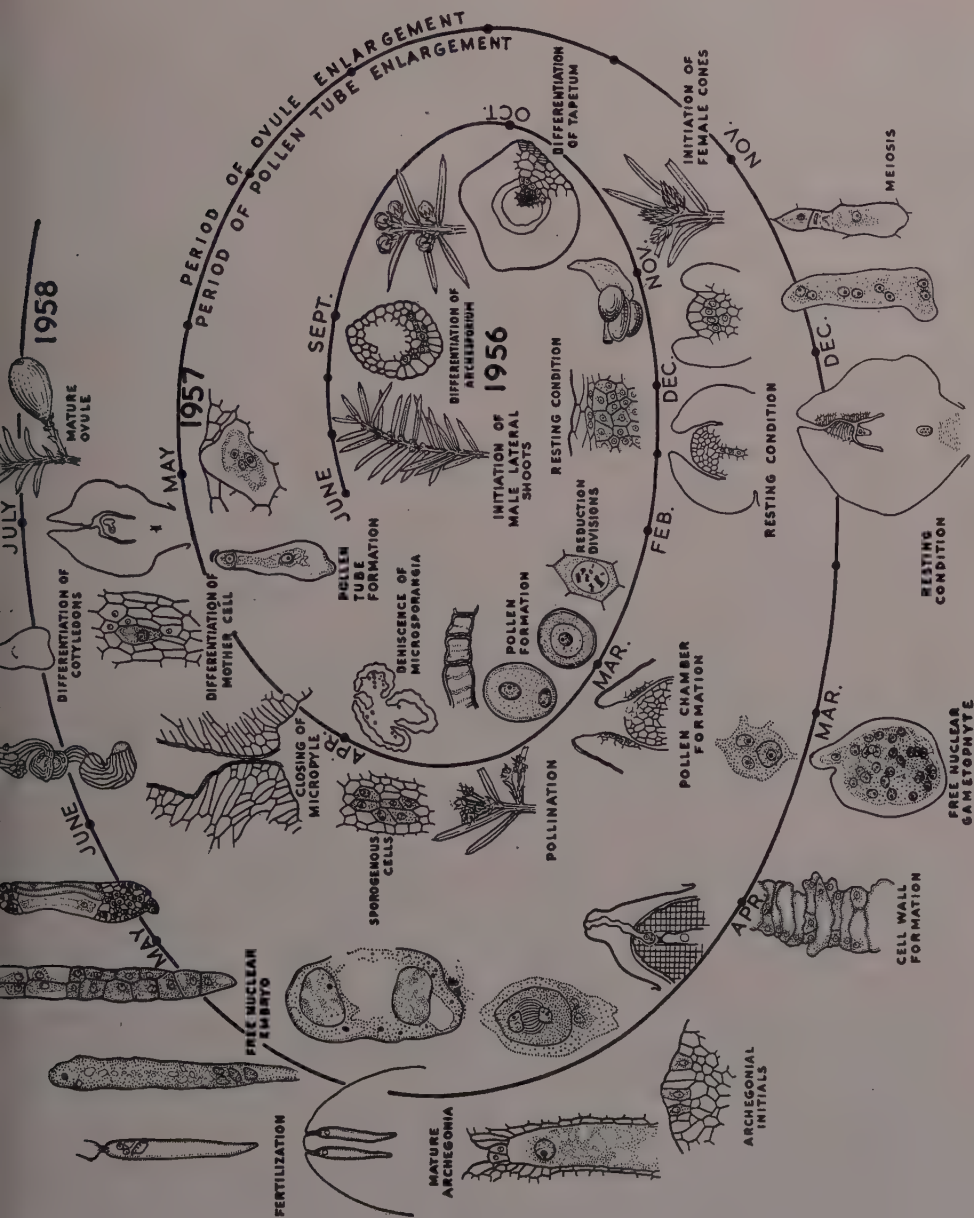
Fig. 23 — Chronology of the reproductive phases. Diagrammatic representation of the life history of *Cephalotaxus drupacea* in relation to the months of a year, as it occurs in Dehra Dun.

of the neck cells. The latter probably abort due to the incoming pollen tube. I have not been able to see any swelling of the egg cell as noted by Arnoldi (1900).

Arnoldi (1900) reported that the pollen tube empties its contents in the fraction of the cytoplasm of the oosphere which protrudes out of the archegonial neck. According to Favre-Duchartre (1957) also, sometimes a portion of the egg cytoplasm is expelled into the archegonial chamber. However, the description and figures given by me hardly leave any doubt that no cytoplasm from the egg gets beyond the archegonial neck and the pollen tube discharges its contents inside the egg cytoplasm.

An open tier has been reported to be absent in the proembryo of Cephalotaxus (see Buchholz, 1925). In my preparations such a tier was clearly seen although it is ephemeral. It is probable that earlier investigators did not have close series of developmental stages. For example, without mentioning a 16-celled proembryo Coker (1907) asserted that the open tier is absent. His Fig. 19 is really a young embryo where the open tier has already degenerated and the prosuspensor tier shows cells formed by transverse divisions. Obviously if one were to look for the open tier at this stage, one is almost certain to miss it.

According to Favre-Duchartre (1957) and Kaur (1958) four tiers, viz. (i) rosette tier, (ii) suspensor tier, (iii) embryonal tier and (iv) ' cap cells ', are formed in the proembryo at the time of wall formation. In my material the ' cap cells ' do not form a separate tier but are merely the lowermost cells of the embryonal group which do not contribute to the formation of the embryo. Two groups, the upper and the embryonal, are formed at wall formation, and not four as reported by earlier authors. The open tier was missed by them although it is easily seen in the earlier stages. Finally a rosette tier is absent. The above mentioned authors seem to have examined embryos of a late stage only in which the prosuspensor had divided to form a group of cells at the top, which they erroneously designated as a rosette tier. In fact a rosette tier does

not occur in any family other than Pinaceae (Doyle, 1957).

In view of the above an alternative explanation is required for the rosette embryos reported in Cephalotaxus (Buchholz, 1925). These are situated in the upper region of the suspensor, and are in fact derived by the divisions of the prosuspensor cells in that region. Coker (1907) figured (his Figs. 19, 20) some cells at the top of the proembryo, but failed to determine their exact origin.

Buchholz (1925) wrote as follows about the proembryos of Cephalotaxus : " Above these (embryonal tier) is a third more even tier of from five to seven or more cells which form the primary suspensor ... ". However, in the proembryo the cells just above the embryonal tier constitute the prosuspensor tier, since the latter is derived from the upper tier of the two-tiered embryo. According to the definition of Buchholz (1939) the prosuspensor forms a *distinct tier of cells in the proembryo* (italics mine), elongating in unison and constituting a specially differentiated part of the entire suspensor system, whereas the primary suspensor is a single elongating cell and is the first such structure to be derived from the embryonal tier. As shown earlier, in Cephalotaxus a suspensor composed of a single elongating cell is never formed, consequently a primary suspensor is absent. In this genus, secondary suspensor directly follows the prosuspensor. Thus Buchholz's (1925) interpretation of the presence of a primary suspensor in the genus is not corroborated.

Lawson (1907) wrote: " After a careful study, I am inclined to believe that these cap-cells originate from a subdivision of the first tier at the tip of the proembryo." The present study indicates that ' cap cells ' are not derived from the division of the embryonal cells but organized at the wall formation stage, although they later degenerate and the adjacent embryonal cells take their place.

Buchholz (1925) considers that " the key to the real homologies between the parts of the embryo of Cephalotaxus and those of pines and other conifers lies in its cleavage polyembryony." This misconception owes itself to the fact he regards the prosuspensor of Cephalotaxus as the

primary suspensor and then compares it with the (true) primary suspensor of other conifers. In fact there can not be any homology between the embryo of *Cephalotaxus* and that of a pine because the two differ basically in the following respects:

(1) The tiers in *Cephalotaxus* proembryo are not so definite and distinct as those of a pine embryo.

(2) The rosette tier and primary suspensor are present in pines but absent in *Cephalotaxus*.

(3) ' Cap cells ' and prosuspensor do not occur in pines but are found in *Cephalotaxus*.

(4) Cleavage of the embryonal mass is very rare in *Cephalotaxus* but is a regular feature in pines.

The proembryo of *Cephalotaxus* shares the following characters with Taxodiaceae, Podocarpaceae and Taxaceae: (i) indistinct tier formation, (ii) absence of a primary suspensor and (iii) the same plan for early embryogeny (*see* Doyle, 1957).

Doyle's (1957) comparison of the embryo of *Cephalotaxus* with that of the podocarps appears to be much more logical than the comparisons which Buchholz (1925) and others have made between the embryo of *Cephalotaxus* and that of the pines.

While describing the vascular transition in the seedling of *C. pedunculata*, Hill & de Fraine (1908) remark, ". . . each xylem mass derived from a cotyledon bifurcates and the protoxylem undergoes a certain amount of rotation, which, however, is not very pronounced . . .". For *C. fortunei* and *C. drupacea* they write, ". . . they differ in no important way from the foregoing species." In my material, however, the xylem patches do not undergo any rotation or bifurcation and the transition to the stem structure is attained by the replacement of centripetal xylem elements by pith and the appearance of centrifugal elements.

According to Favre-Duchartre (1957) meiosis in the microspore mother cells begins on December 8, microspores are formed by February 16, and pollen matures by March 13. In my material December 8 to February 16 is the resting period. Paris (49° latitude) from where

he collected the material has a more temperate climate than that of Dehra Dun (30·5° latitude) from where I collected my material. It is surprising that while the mother cells are in a resting phase during December-February in a less temperate place (Dehra Dun), in the same period they undergo reduction divisions in a cooler place (Paris). However, it takes only 10-15 days for the formation and maturation of pollen grains at Dehra Dun, while almost a month is required for the same at Paris. It seems that Favre-Duchartre (1957) did not examine all the stages of pollen formation.

According to all the previous authors the ovule passes through just one' winter, whereas, the present work clearly shows that it passes through two winters. Apparently the previous workers failed to see the ovules of the first year which remain hidden in the bud scales. The only winter in which they have described the ovule as resting is evidently the second winter.

Arnoldi (1900) reported that the megaspore mother cell in an ovule differentiates one year after pollination and the female gametophyte starts developing about two months later. So according to him the ovule contains only sporogenous cells even in the second winter. Coker (1907) differed from Arnoldi (1900) and stated that in the second winter the ovule contains a megaspore, i.e. the mother cell undergoes meiosis before the resting period is reached. Lawson (1907) wrote that the ovule passes the second winter in the mother cell stage and meiosis takes place one year after pollination. Favre-Duchartre (1957) has agreed with Lawson (1907) and according to him the mother cell differentiates six months after pollination, and during the second winter the ovule rests in this stage. The mother cell undergoes meiosis only in the beginning of the spring (middle of February). The present work clearly shows that the mother cell differentiates just one month after pollination; it undergoes meiosis and a free nuclear female gametophyte with 8-16 nuclei is formed before the second winter approaches. It seems that previous workers did not make continuous collections at frequent intervals. It

appears also that some of the sections studied by them were not median. For example, Fig. 3 of Lawson (1907) clearly represents a tangentially cut ovule.

TAXONOMIC RELATIONSHIPS — Conventionally *Cephalotaxus* is considered to be related to podocarps or taxads or occupying an isolated position among the living conifers. The three views are being discussed here separately.

Relation with Podocarps — Sinnott (1913) pointed out that the vascular system of the female cone of *Cephalotaxus* resembles that of the Podocarpineae, especially *Phyllocladus*. The fleshy integument of *Cephalotaxus* was compared to the seed coat (consisting of the fused fleshy epimatium and the integument) of *Podocarpus*, sub-genus *Stachycarpus*. Sinnott further wrote: " In the structure of the ovule also, *Cephalotaxus* shows a strong resemblance to *Podocarpus*, especially the sub-genus *Stachycarpus*." After finding many such similarities he concluded that *Cephalotaxus* provides an intermediate condition between *Podocarpus* and the Taxineae. Similar conclusions were reached by Bliss (1918) on the basis of wood anatomy. From a study of embryogeny alone, Buchholz (1920) showed that *Cephalotaxus* is related to *Podocarpus*.

Already Pilger (1903), Brooks & Stiles (1910) and Stiles (1912) had expressed the view that there is little evidence of a close connection between the Taxineae and the Podocarpineae. In his paper on the sturcture and affinities of *Acmopyle pancheri*, Sahni (1920) remarked that *Taxus, Torreya* and *Cephalotaxus* are structurally so distinct that they have nothing in common with the podocarps and that they should be given the rank of a separate order, the Taxales. He removed *Acmopyle* from the Taxineae to the Podocarpineae. Wilde (1944) working on the sturcture of the cones of the Podocarpaceae emphasized the distinctness of the family. Thus presently there is hardly any doubt that *Cephalotaxus* has no relationship with podocarps. However, Takhtajan (1953) speaks of a close relationship between the Taxaceae, Cephalotaxaceae and Podocarpaceae. This probably stems mainly from his incorrect interpretations of the micro-

sporophylls of *Cephalotaxus* which were supposed to bear pendant sporangia.

Relation with Taxads — For a long time *Cephalotaxus* has also been treated as a taxad. This was probably due to the presence of tertiary spiral thickenings in the xylem tracheids (a feature common to all taxads) and an imperfect knowledge of its female cones. Its mature fleshy seed was compared with that of the taxads. The vegetative parts of the plant are also similar. Pilger (1903) gave a fairly correct description of the female cones of *Cephalotaxus*, but did not consider a transference of the genus to a new family. Neger (1907) for the first time created the family Cephalotaxaceae containing the only genus *Cephalotaxus*. Pilger (1916, 1926) supported Neger's (1907) view on the erection of the Cephalotaxaceae but included a new genus *Amentotaxus* under this family. Pilger (1926) described the female cone of the family as follows: " ♀ Blüten in den Achseln von Schuppen am Grunde von später auswachsenden Zweigen, kurz gestielt, mit mehreren gekreuzten Paaren von Carp., diese mit 2 Samenanlage . . .". This description matches quite well with the structure of the female cone of *Cephalotaxus*. However, the 'flower' of *Amentotaxus* was described by Pilger (1926) as follows: " ♀ Blüte wenig bekannt, in der Achsel einer Braktee am Grunde eines beblätterten Zweiges sitzend, kurz dicklich gestielt, Samenanlagen ? " Since then our knowledge of *Amentotaxus* has been supplemented by Florin (1931, 1938-45) who has shown that it has typical taxoid female ' flowers ', i.e. the ovules are borne singly and appear terminal on the floral axis. In *Cephalotaxus*, on the other hand, the ovules are borne in cones and are lateral on the floral axis. Florin has also worked on the stomatal structure of the two genera and has pointed out that the stomatic bands in *Amentotaxus* are characteristically thickened in comparison with the non-stomatiferous areas, but unthickened in *Cephalotaxus*. Further, in arrangement and structure also the stomatal apparatuses differ. The epidermis of *Amentotaxus* is unique among living conifers and taxads and a single leaf fragment is sufficient to prove that it

represents a new genus. Thus in structure of the female cone and stomata the two genera clearly stand apart from one another. Florin (1948) transferred *Amentotaxus* to the Taxineae and Pilger & Melchior (1954) have followed this in the latest edition of the " Syllabus der Pflanzenfamilien ". Although a detailed investigation is still awaited, it is apparent that *Amentotaxus* is not related to *Cephalotaxus* and there is no justification for putting the two in one family. A table comparing the Cephalotaxaceae (containing the only genus *Cephalotaxus*) and Taxaceae (containing the five genera *Taxus, Torreya, Austrotaxus, Amentotaxus* and *Pseudotaxus*) is given below:

TABLE 1

CEPHALOTAXACEAE	TAXACEAE
Leaves are arranged in an opposite and decussate fashion	Leaves are arranged in a spiral manner
Tertiary spiral thickenings present in the xylem tracheids, xylem parenchyma diffused	Similar to the Cephalotaxaceae
Microsporophylls hyposporangiate	Microsporophylls perisporangiate
Two ovules are present laterally on a short axis which is borne in the axil of each bract of a female cone	Ovules borne singly and terminally on short fertile shoots
Aril absent	Aril present
The ovule is supplied by two inverted bundles	The ovule is supplied by a variable number of normally oriented bundles
Prothallial cells are absent in the pollen grains which are shed at the two-celled stage	Similar to the Cephalotaxaceae, but shed at the one-celled stage
Pollen grains unwinged	Pollen grains unwinged
Pavement tissue present in the ovule	Pavement tissue absent
Wall formation in the female gametophyte takes place by centripetally advancing cells	Wall formation takes place by alveoli
A primary suspensor is absent, the embryonal mass does not undergo any cleavage and ' cap cells ' are present in the embryo	A primary suspensor is absent, the embryonal mass shows cleavage, ' cap cells ' are absent
Mature integument fleshy	Mature integument not fleshy

Thus it is apparent that Taxaceae and Cephalotaxaceae resemble each other in wood structure, pollen structure and to some extent embryogeny. However, tertiary spiral thickenings in the xylem tracheids are not restricted to these two families only but occur in several genera (like *Picea, Pseudotsuga, Larix*) of the unrelated family Pinaceae. Unwinged pollen grains without prothallial cells occur as a rule in Cupressaceae and Taxodiaceae also. Embryogeny similar to that in the above two families is also found in Podocarpaceae, Taxodiaceae and Cupressaceae. Therefore, the resemblances between the two families are not such as to indicate any close affinities. On the other hand, they differ basically in the arrangement of leaves, structure of the microsporophylls, structure and position of the ovule, wall formation in the female gametophyte, structure of the mature integument, and to some extent in embryogeny. Florin (1954) has laid special emphasis on the differences between the reproductive organs of the taxads and the other conifers, and it appears best to regard the Taxaceae and the Cephalotaxaceae as unrelated.

Isolated Position of Cephalotaxus — This view has been proposed and supported by Florin (1938-45, 1948). His views are based mainly on the female cone structure. According to him the female cone of *Cephalotaxus* does not resemble that of any living conifer but can be derived from the extinct mesozoic genera like *Palissaya* and *Stachytaxus*. However, *Cephalotaxus* resembles the living conifers in seedling structure, leaf trace anatomy, hyposporangiate microsporophylls, biaxial female cone, open tier in the proembryo, and basal plan of embryogeny. In the face of these similarities it is difficult to think of this genus as related only to some mesozoic forms of conifers and quite isolated from the living ones.

Considering all the differences and similarities of *Cephalotaxus* with the conifers, it appears best to put it in a monogeneric family under the order Coniferales.

Summary

Cephalotaxus drupacea is strictly dioecious and under cultivation grows as a

small tree in Dehra Dun. The leaves are linear and acute and arranged in decussate and distichous fashion.

The female cones emerge in bunches of three to five from the resting buds and are borne in the axils of reduced foliage leaves. They are small, short-stalked, and green at the time of emergence. Each consists of five to seven pairs of opposite and decussate bracts. Two ovules with a small secondary axis in between, are present in the axil of each bract. Generally only one or two ovules mature per cone. The seeds are large and become red and fleshy on ripening.

The male cones are sessile and arise in compact groups of six to eight, on short unbranched lateral branches in the axils of most of the leaves of the new shoots. Each bears 15-20 spirally arranged, hyposporangiate microsporophylls.

The short lateral shoots which bear the male cones are supplied by a ring of vascular tissue formed by the fusion of two sickle-shaped bundles. A sterile bract is supplied by a single unbranched bundle. Three bundles supply each unit of the fertile bract and axillary male cone. One vascular trace departs for each microsporophyll.

Three bundles supply the fertile leaf and the axillary female cone, as also a bract and the axillary ' seed-scale complex '. Two inverted bundles supply each ovule. Anatomical evidences have been given to show that the female cone is biaxial.

A plate of hypodermal cells differentiates on the abaxial surface near the base of each microsporophyll. Later, due to sterilization at places, two to four groups of archesporial cells are delimited, each becoming the seat of a microsporangium. The sporogenous cells are surrounded by four wall layers and the epidermis. The innermost wall layer forms the tapetum and its cells become binucleate at the microspore mother cell stage. At maturity the epidermis develops fibrous thickenings while the remaining wall layers are crushed. The sporangia dehisce along a line facing the stalk of the microsporophyll.

The microspore mother cells are full of starch. At the time of the reduction divisions their cytoplasm contracts from the wall and a special mucilaginous wall is secreted. Twelve bivalents could be counted at diakinesis. Wall formation takes place after Meiosis II. Both tetrahedral and isobilateral types of microspore tetrads are present. Several abnormalities of microsporogenesis have been observed. At the time of shedding, the pollen grains contain a tube and an antheridial cell. The prothallial cells are absent. The exine of the pollen seems to be cast off while the pollen is caught in the pollination drop.

On the nucellus the pollen grain produces a short unbranched tube. The antheridial cell divides to form the body and the stalk cells. The latter soon loses its wall so that later on only a stalk nucleus is seen. In the following year the tube grows rapidly. It reaches the female gametophyte when the archegonia are almost mature and the body cell divides to form two male nuclei of equal size. The stalk and tube nuclei degenerate by this time and the pollen tube discharges the male gametes inside the archegonium.

Three to six hypodermal archesporial cells are present in an ovule. They divide to form the sporogenous and the primary parietal layers. The latter produces a massive nucellus. A ' pavement tissue ' is present at the chalazal end of the ovule. A rudimentary pollen chamber is formed at the top of the nucellus. After pollination the micropylar canal becomes closed due to the ingrowth of the cells of the inner epidermis of the integument.

Out of the many sporogenous cells, only one enlarges and functions as the megaspore mother cell. A linear tetrad of megaspores is formed. The chalazal megaspore functions and produces a free nuclear gametophyte having a short finger-like projection at the micropylar end. Wall formation in the gametophyte takes place by centripetally advancing cells.

Three to five archegonial initials differentiate at the micropylar end of the cellular gametophyte. They divide periclinally to give rise to the neck initial and the central cell.

The division of the central cell is not followed by wall formation so that only a

ventral canal nucleus (which is ephemeral) is formed. Occasionally the nucleus of the central cell functions directly as the egg nucleus. The mature archegonium comprises a two-celled neck and a long narrow egg. Each archegonium has its own jacket layer.

The various stages of fertilization have been recorded.

The first division of the zygote nucleus is intranuclear and occurs *in situ*. The resulting two nuclei migrate to the base of the archegonium. Wall formation in the proembryo takes place at the 16-nucleate stage.

A varying number of large nuclei, which degenerate at later stages, are seen in the upper region of the proembryo. The different sources of their origin have been traced.

Two groups are organized after wall formation in the proembryo of which the lower is the embryonal group. The lowest cells of the latter are very large and constitute the ' cap cells '. The other tier divides further to form the uppermost tier, which is open towards the archegonium; and the prosuspensor. Both the open tier and the ' cap cells ' degenerate early. Before elongation the prosuspensor tier cuts off cells at the top which develop into small abortive embryos.

A primary suspensor is absent, but the secondary suspensor is very massive. A cleavage of the embryonal mass is rare. The mature embryo has two cotyledons and at the time of seed shedding root initials are well differentiated. The shoot apex is organized only at the time of germination of the seeds.

The seed coat is very thick and consists of an outer fleshy, a middle stony and an inner papery layer.

The germination of the seeds is of the epigeal type. The juvenile leaves are spirally arranged and the cotyledonary leaves persist for more than two years after germination. The hypocotyl of the seedling shows root structure. The transitional zone between the root and the cotyledons shows a replacement of the centripetal xylem tracheids by pith and the appearance of centrifugal xylem. The cotyledonary bundles are associated with a few short centripetal xylem tracheids which are also present in the juvenile leaves.

A chronological study of the reproductive phases has been made.

Cephalotaxus should be placed in a monogeneric family Cephalotaxaceae. The family does not seem to be allied to Podocarpaceae or Taxaceae. It resembles the living conifers in several important features and should be included in the order Coniferales.

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AGNES ARBER

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Dr Agnes Arber, F.R.S., F.L.S., famed for her contributions to plant morphology and for her philosophical exposition of biology, died in Cambridge on March 22, 1960. She was in her eighty-second year, and she retained her mental vigour to the last. She is buried in Girton churchyard with her husband E. A. N. Arber, the palaeobotanist, who died in 1918. Mrs Arber, as she was generally known, came from a scholarly and artistic family, and these qualities pervade her work. Beginning with the " Herbals " (1912) and then " Water Plants " (1920), " Monocotyledons " (1925), " Gramineae " (1934), and ending with " The Natural Philosophy of Plant-Form " (1950) and " The Mind and the Eye " (1954), the reader may revel in living phytomorphology. Her technique was simple, that is by orthodox transverse sections, but her reconstitution of what the eye saw was consummate. She represents what may be called the escarpment of morphology. It is an immense front of knowledge abruptly giving place to, not overlain by, the new rise of biochemical morphogenetics, which began too late for her appraisal. Her friend and

teacher, Miss Ethel Sargant, doubtless lead her to the monocotyledons, than which there are few more gracious plants, for botanists are most human scientists, as can be gathered from Mrs Arber's writings, and their subjects reflect their ownselves. It is a pity that she chose to become a recluse, to set aside academic vocation, and to work privately in her home on Huntingdon Road. Though many came from abroad to visit her, she was almost unknown to the majority who passed through the nearby Botany School. Yet, in its library no books are more thumbed than hers, and, when told of this in her last days, she wrote that her heart began to throb again. Mrs Arber was elected to the fellowship of the Royal Society in 1946. In 1948 she was awarded the Linnean Gold Medal, and she had been a Fellow of the Linnean Society of London since 1908. Of her works and of her career Dr H. Hamshaw Thomas has written in detail (Nature, June 11, 1960). She was a Life Member of the International Society of Plant Morphologists. A graduate of Newnham College, Cambridge, she went as Miss
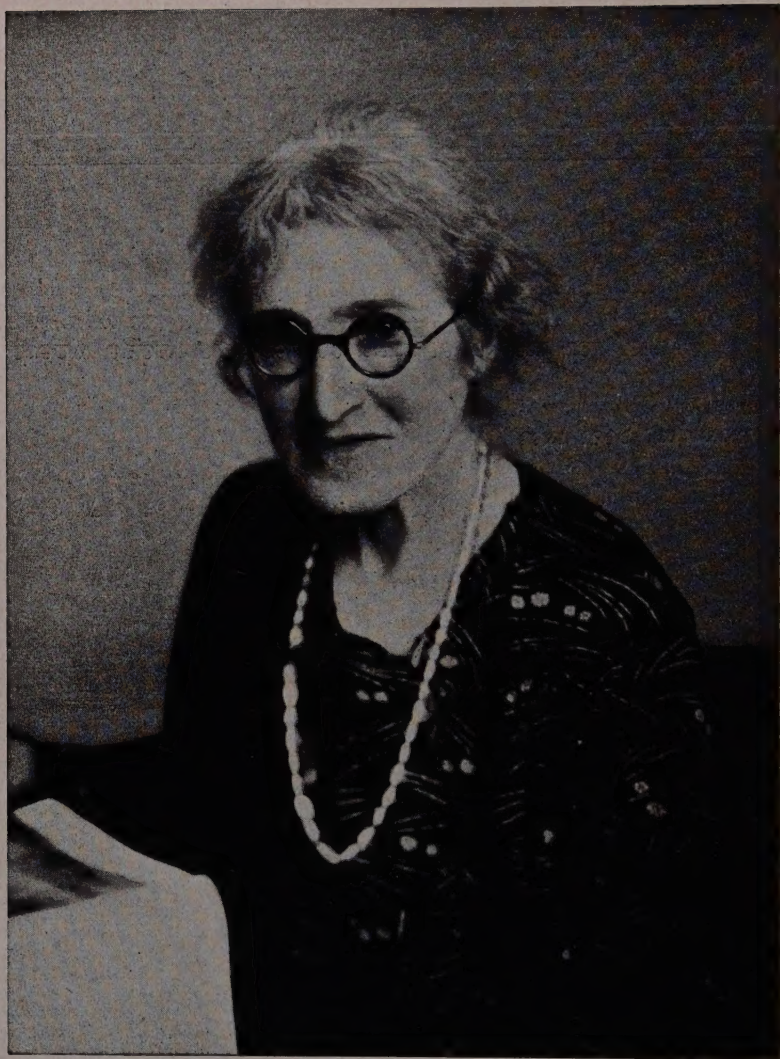
Dr Agnes Arber, F.R.S., F.L.S.

Robinson for some years to University College, London, and returned to Cambridge as Mrs Arber in 1909. There she found the full scope for her scholasticism, powerful, brilliant, and humorous, and, if |such a secret may be divulged, her favourite prayer was that of Ovin " the faithful thane of St. Ethelreda, which has stood since their day in Ely Cathedral, roughly hewn on his commemoration stone: Lucem tuam Ovino da Deus et requiem ".